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ABSTRACT

Title of dissertation: Cloning, Characterization, and Functional Analysis of Murine Pregnancy Specific Glycoproteins

Jennifer L. Wessells, Doctor of Philosophy, 1999

Dissertation directed by Gabriela S. Dveksler, Ph.D.

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Pregnancy Specific Glycoproteins (PSG) are a group of highly conserved placental proteins that are present at high concentrations in the serum of pregnant women. As a result, these proteins are postulated to have a critical role in maintaining a successful pregnancy. A murine model was established to examine whether recombinant PSGs regulate the expression of cytokines by macrophages, a cell type known to be prevalent in the uterus during pregnancy. To this end, full length *Psg18* and *19* cDNAs were cloned, and the anatomical sites of *Psg17*, *18*, and *19* expression determined. The baculovirus expression system was used to generate recombinant PSG17, 18, 18N, and 19 as fusion proteins. The full length proteins were generated as GST fusion proteins and the PSG18N, a truncated form of PSG18 containing only the N1-domain, was generated as GST and 6x His fusion proteins. Since no currently available antibody shows reactivity with murine PSGs and human anti-PSG antibodies do not demonstrate crossreactivity with the mouse proteins, a polyclonal antibody was generated to PSG18N. To determine the functional role of these proteins, the recombinant murine PSG18

and PSG18N were used to investigate whether PSGs regulate cytokine expression by thioglycollate-stimulated peritoneal macrophages and the murine macrophage cell line RAW 264.7. Both PSG18 and PSG18N were found to induce murine macrophages and RAW 264.7 cells to produce IL-6 and IL-10 mRNA. At the protein level, PSG18N stimulates both cell types to secrete of IL-6 and IL-10 in a dose dependent manner. In addition, the PSG synergized with LPS to induce secretion of IL-6 and IL-10 protein. The affect of PSG18N treatment on expression of IL-1β, TNF-α, TGF-β, iNOS, and IL-12p40 mRNA in RAW 264.7 cell and C3H/HeJ macrophages was also examined. My data show that PSG18N did not alter the expression of these immune mediators at the mRNA level. Collectively, these results indicate that PSGs specifically upregulate cytokines that are known to be helpful to pregnancy. PSGs function to regulate the maternal immune system, thereby generating the anti-inflammatory environment which is necessary to ensure survival of the fetal allograft.

CLONING, CHARACTERIZATION, AND FUNCTIONAL ANALYSIS OF MURINE PREGNANCY SPECIFIC GLYCOPROTEINS

By

JENNIFER LYNN WESSELLS

Dissertation submitted to the Faculty of the Department of Pathology Graduate Program of the Uniformed Services University of the Health Sciences in partial fullfillment of the requirement for the Degree of Doctor of Philosophy, 1999

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I. Introduction

Pregnancy Specific Glycoproteins

Significant physiologic changes occur during pregnancy. Proteins produced by the placenta mediate part of these changes. These proteins include the pregnancy specific glycoproteins (PSGs) which are a group of highly conserved proteins originally isolated from the retroplacental circulation of pregnant women (1, 2). Pregnancy-specific glycoproteins are also known as pregnancy-specific β1-glycoprotein ($PS\beta_1G$), pregnancy β1-globulin (PBG), Schwangerschaftsprotein1 (SP_1), pregnancy-associated plasma protein-C (PAPP-C), and trophoblast-specific β1-glycoprotein (TSG) (1-3). In humans, PSGs are a family of proteins of unknown function that are synthesized by the syncytiotrophoblast cells and are secreted into the maternal circulation (1, 4). There is no available data on the amino acid sequence of mature PSGs, but it is clear that multiple PSG species are present in the circulation of pregnant women (3). They are detectable in the serum of pregnant women as early as two weeks post-conception (5). The concentration of PSGs increases exponentially during pregnancy, reaching 200-400 μg/ml at term (6). Within 24-48 hours of delivery, PSGs are no longer detectable in the bloodstream (7). The high concentration of PSGs suggests that they have a critical role in pregnancy. In addition, low levels of PSGs are common to certain pathological conditions such as spontaneous abortion (8, 9), intrauterine growth retardation (10), fetal hypoxia (11), and pre-eclampsia (12). Furthermore,

treatment of pregnant monkeys (13) and mice (14) with antibodies against PSGs results in a high rate of abortion.

PSGs have also been identified from extra-placental sources. Low levels of PSGs, less than 0.5 μg/ml, are detectable in normal, healthy, non-pregnant individuals (15, 16). Increased expression of *PSG* mRNA has been detected in human adult colon (17, 18), testes (19, 20), and fetal liver (21). In addition, elevated levels of PSGs are found in the serum and tissues of women with gestational trophoblastic tumors and trophoblastic diseases, including choriocarcinomas, chorioepithelioma (22), hydatiform moles (23), and invasive moles (24). In fact, PSG levels have been utilized to monitor the treatment of trophoblastic tumors (25).

❖Relationship to Immunoglobulin Superfamily

PSGs have a high degree of sequence similarity with members of the carcinoembryonic antigen (CEA) gene family (26). Based on the amino acid sequence comparison, the CEA gene family is divided into two main subgroups: the CEA subgroup consisting of non-specific cross-reacting antigens (NCA), biliary glycoproteins (BGP), and carcinoembryonic antigens (CEA), which are mostly membrane-bound and the pregnancy specific glycoproteins (PSG) subgroup consisting primarily of secreted proteins (27). Together *PSGs* and CEAs form a subfamily of the immunoglobulin (Ig) gene superfamily (28-31). Human PSGs and CEAs share 70 % nucleotide and 55 % amino acid identity (32, (32).

The conservation of amino acid and nucleotide sequences suggests that the two gene families evolved through duplication of one primordial gene (33).

❖Gene Structure of Human and Murine *Psgs*

According to the "Genetic nomenclature guide" by Trends in Genetics 1998, the following nomenclature has been generated to describe human and murine Pregnancy-Specific Glycoproteins. For both human and murine proteins, the letters are all capitalized (PSG). The gene names are always italicized with capital letters used for the human (*PSG*) and lowercase letters used for the mouse (*Psg*).

PSG cDNAs have been cloned from the human, mouse and rat placenta (34, 35). Human PSGs and murine PSGs possess an N-terminal domain of similar structure. The N-terminal domains of rodent and human *PSGs* share approximately 50% similarity at the nucleotide level (36). However, there are significant differences in the domain organization of human and murine *Psgs*. There are eleven human *PSG* genes that are clustered on chromosome 19q13.1-13.3, adjacent to the CEA subgroup genes (37, 38). All human *PSG* genes possess a similar domain organization (27). The proteins consist of a 34 amino acid leader sequence (L), a 108 amino acid N-terminal domain (N) that resembles the immunoglobulin (Ig) variable domain, two to three similar Ig constant-like domains, and a 2-81 residue, hydrophilic C-terminal domain (C). Each constant-like domain consists of a 92-residue A-domain or a 85-residue B-domain and contains at least 2 cysteine residues that are thought to stabilize Ig-like folding via a disulfide bridge (39). As a result, the domain organization of the majority of the

human *PSG* cDNAs is L-N-A1-A2-B2-C, L-N-A1-B2-C, or L-N-A2-B2-C (39, 40). The 11 human *PSG* genes encode more than 30 different gene products generated by alternative splicing (41). Human PSGs have 6-7 potential N-linked glycosylation sites, and when fully glycosylated they have an estimated molecular weight of 90 kDa (27, 28, 42).

The 14 murine *Psg* genes are mapped to a region on chromosome 7 that is synteneic to human chromosome 19 (43, 44). Full length cDNA for *Psg17* (formerly named Cea 2), and partial cDNAs for *Psg18* and *Psg19* (formerly named Cea 3 and Cea 4) were obtained from a 13 day mouse placental cDNA library (35). In addition, partial genomic sequences were obtained for the remaining murine *Psgs*, but the corresponding cDNAs have not yet been cloned (35, 45). Murine PSGs are composed of a 34 amino acid leader peptide followed by three Ig variable-like or N-domains (105-120 amino acids each) and a single 93 amino acid Ig constant-like or A domain (35). The N-domains have approximately 55 % or 45 % similarity at the nucleotide and amino acid level, respectively. In addition, the N-domains are separated by 12 amino acid leader fragments (L'2, L'3) that contain 1 or 2 cysteine residues. Similar to the human proteins, murine PSGs possess 6-7 N-linked glycosylation sites and are capable of generating a fully glycosylated protein with an estimated molecular weight of 70 kDa (35). The carbohydrate moieties, consisting primarily of glucosamines, comprise approximately 30 % of the total PSG mass (28)

❖Potential Biological Activity of PSGs

Despite their sequence similarity, PSGs and CEAs are not believed to share the same biological activities. CEAs are cell surface proteins that have various functions, including intercellular adhesion and bacterial binding. Additionally, they are an oncofetal antigens which are expressed at very high levels in epithelial carcinomas such as colon cancer and other adenocarcinomas (18, 46). It has been suggested that PSGs may have a role in mediating cellular interaction between cell surface receptors and extracellular matrix proteins (26). The human PSG 2, 3, 5, 6, 7, 11, and 13 contain an arginine-glycine-aspartic acid (RGD) motif. Murine PSGs possess a RGD-like motif within their N1-domain. The RGD-like motif consists of a positively charged amino acid (Arg, His) followed by a glycine and a negatively or positively charged amino acid (Lys, Glu). This motif constitutes a portion of the cell binding domain of many extracellular matrix proteins which bind cell surface receptors that belong to the integrin family (47, 48). Fibronectin, collagen, and vitronectin all possess this integrin binding motif (49, 50). The presence of the RGD or RGD-like sequence may have an important role in the function of PSGs.

Previous reports suggest that PSGs may function to prevent the immune rejection of the fetus, or to regulate trophoblast invasion of the uterus, resulting in the maintenance of a normal pregnancy (32). In support of this hypothesis, Mansfield and co-workers have shown that both PSGs isolated from the placenta

and a peptide derived from the N-terminal domain of PSG11 possess the RGD motif and binds to human monocytes and cells of the promonocyte lineage but not to T or B cells (51). These results indicate that human PSGs may function by binding to monocytes or macrophages. More recently, human PSG11 has been shown to stimulate monocytes to secrete the anti-inflammatory cytokine, IL-10 (52). Although human and murine PSGs are likely to have similar biological activities, genes encoding murine *Psgs* are not well characterized, and consequently their functional roles remain unknown.

Implantation and Placentation

Survival and development of a successful pregnancy are very complicated and finely programmed processes. A synchronized sequence of events during pre-implantation is critical for the proper development of the implanted embryo (53). Successful implantation requires that the embryo develops into a blastocyst while steroid hormones prepare the uterus for invasion (54). The blastocyst differentiates into an outer layer that consists of the trophoblast and an inner layer called the inner cell mass. The outer layer forms the portion of the placenta that is in direct contact with maternal blood. The inner layer develops into the embryo, amnion, and the mesenchmal and vascular tissues of the placenta (55-57). Implantation occurs at 3.5 to 4 days post coitus (d.p.c.). The invasion of the uterine epithelium and underlying endometrial stroma by extraembryonic trophoblast cells ultimately results in the formation of the placenta, an endocrine

organ that consists of both embryonic and maternal tissues. The placenta arises from the collaboration of the extraembryonic mesoderm, cytotrophoblast, and syncytiotrophoblast cell layers of the fetus with the endometrium of the uterus (58). Syncytiotrophoblast cells are responsible for the initial invasion of the endometrium, while deeper invasion is accomplished by the cytotrophoblast (54). The maternal portion of placenta arises from the functional layer of the endometrium called the decidua and the fetal portion develops from the chorionic sac. Uterine stromal cells on the mesometrial side of the uterus differentiate and proliferate into decidual cells (54). The maternal part of the placenta develops from a portion of the mesometrial decidua called the decidua basalis (59). The decidua functions to control invasion of the trophoblast and provide nutrients to the developing fetus.

Following implantation, the placenta must be rapidly generated in order for physiological exchange to occur between the maternal and fetal tissues (60). Survival of the mammalian embryo depends on the uncompromised supply of oxygen and nutrients by the mother. The trophoblast invasion that occurs during implantation replaces the walls of maternal arteries and veins with trophoblast cells, forming the vasculature of the placenta (61, 62). This process anchors the placenta to the uterus and supplies blood to the fetus by way of the intervillous pool. Maternal blood within the intervillous space circulates around the projections of fetal tissue from the chorionic plate, called chorionic villi, allowing for the exchange of gases, metabolites and waste between maternal and fetal

circulations. Chorionic villi consist of a syncytiotrophoblast cell layer and a cytotrophoblast layer that overlay a vascular mesoderm core. Survival of the fetus depends on the adequate bathing of chorionic villi by maternal blood. Reduction in the uteroplacental circulation results in fetal hypoxia and growth retardation (63, 64).

Survival and development of the mammalian conceptus depends on several factors, including prevention of luteolysis, suppression of mechanisms of immunological rejection, and development of a means for the exchange of metabolic substances and waste products (62, 65, 66). Rats and mice are among the few laboratory species that share a similar process of chorio-allantoic placental development with the human system (60). Both rodents and humans possess hemochorial placentation, meaning that the trophoblast cells derived from the trophoectoderm layer of the blastocyst gain direct access to the circulating maternal blood (67). In addition, ovarian steroidal hormones, such as estrogen and progesterone, regulate the estrous cycles of rats and mice, and the menstual cycle of humans.

As is evident from the brief description above, placentation and implantation are complex and highly regulated processes. Trophoblast invasion of the uterine wall is critical for a successful pregnancy, but it must be tightly controlled in order to prevent complications such as intrauterine growth retardation and trophoblastic tumors. In human preganacy, decidual and trophoblast cells secrete transforming growth factor β (TGF-β) and

cytotrophoblast cells secrete interleukin 10 (IL-10) into the extracellular matrix. TGF-β functions to control invasion by promoting differentiation of the cytotrophoblast and subsequent upregulation of metalloproteinase inhibitor production by the cytotrophoblast. Trophoblast and decidual cells are stimulated to produce tissue inhibitor of metalloproteinases (TIMP) that functions to inactivate type IV collagenases, preventing further degradation of the decidua (68-70). In addition, IL-10 is secreted by the cytotrophoblast and functions to downregulate secretion of matrix metalloproteinase 9 (MMP-9), an extracellular matrix (ECM)-degrading enzyme, in an autocrine manner (71).

During invasion, interleukin 1β stimulates the cytotrophoblast to express MMP-9 (72). The observations of Roth and co-workers also suggested that expression of MMP-9 and TIMP are regulated by different mechanisms (71). Pregnancy complications such as pre-eclampsia (73) and intrauterine growth retardation (74) are associated with shallow cytotrophoblast invasion. One possible cause of these complications could be misregulation of expression of matrix MMP-9, a molecule reported to be critical to invasion, by the differentiating trophoblast (72, 75). Although, the exact means of control are not clear, as described above cytokines and growth factors are known to have a critical role in the process of trophoblast invasion (58).

The Unique Immune Environment of the Uterus During Pregnancy

Within the intervillous spaces of the placenta, maternal blood comes in direct contact with fetal trophoblast cells that express paternal alloantigens (76). Maternal and fetal cells are intermingled on the maternal side of the intervillous space (77). Since major histocompatability (MHC) antigens are necessary for immune recognition and subsequent rejection of grafts, their pattern of expression by fetal trophoblast cells is tightly regulated. According to the theories of tissue transplantation, the mother's immune system should elicit a response to paternal antigens on the surface of fetal cells, resulting in rejection of the conceptus. Maintenance of a successful pregnancy requires the development of an environment that precludes immune rejection of the semi-allogeneic fetus by its immunocompetent mother. Several theories have arisen to address this unique immunological phenomenon. Medawar first suggested the following mechanisms to explain the paradox of fetal survival: (1) immunologic tolerance of the mother; (2) antigenic immaturity of the fetus; and (3) anatomic separation of the mother and fetus (78).

In humans, maternal antibodies to paternally derived placental HLA antigens are detectable in the circulation of multiparous women but do not bind to the surface of syncytiotrophoblast cells (79, 80). In both mice and humans, subpopulations of trophoblast cells differentially express Class I MHC antigens, but do not express Class II MHC antigens (81, 82). During allogeneic tissue rejection, MHC Class I molecules present antigens to the T cell receptor (TcR) of

CD8 positive, cytotoxic T cells. Certain subpopulations of trophoblasts cells lack Class I antigens. Much of the early research suggested that the Class I positive fetal cells were not directly exposed to maternal circulation (83). For humans, reports indicate that the invasive cytotrophoblast does not express the classical MHC Class I genes, rather it expresses the non-classical, Class I gene HLA-G (84-86). The HLA-G is selectively expressed at the fetal-maternal interface and can form classical MHC class I-like complexes (87). The question of whether mice possess a counterpart to the human HLA-G remains to be answered. The HLA-G may be a universal "self"-transplantation antigen that functions to help prevent rejection of the feto-placental unit by the maternal immune system (85, 88). Recently, HLA-G has been shown to prevent the destruction of fetal cytotrophoblast by natural killer (NK) cells (89-91).

The complex nature of pregnancy suggests that there are probably multiple mechanisms involved in preventing rejection of the semi-allogeneic fetus. Studies performed by Munn and co-workers indicate that the trophoblast down regulates maternal T cell responses by catabolizing tryptophan. Their work showed that increased expression of indoleamine 2,3-dioxygenase (IDO) and decreased levels of circulating tryptophan are associated with the normal progression of pregnancy (92). IDO is a tryptophan catabolizing enzyme that is produced by macrophages and the syncytiotrophoblast. These results suggest that the semi-allogeneic fetus actively defends itself against attack by the maternal immune system. In another system, Sacks and co-workers propose that the

mother's innate immune system has a role in maintaining pregnancy (93). The innate immune system has a central role in determining which antigens will be presented to the acquired immune system and which antigens will not be recognized as foreign by cells of the immune system (94). They hypothesize that placental products, presumably pregnancy specific factors, result in the activation of the maternal innate immune system (93). In addition, the report indicates that placental products target monocytes, not lymphocytes, stimulating the secretion of cytokines, enzymes, and lipids known to be involved in generating an anti-inflammatory uterine environment (93). Activation of the innate immune system, along with several other protective mechanisms, could to generate an environment that favors success of the feto-placental unit.

Cells, Cytokines and Pregnancy Success

Investigation of the immunoregulatory molecules present at the fetal-maternal interface has been the focus of much research in the past several decades. Cytokines have important roles in various stages of pregnancy, including implantation and delivery (95). Studies of pregnancy using mice that possess mutations in genes encoding cytokines provide evidence that cytokines are involved in the reproductive process. Female *op/op* mice that possess a mutation in their macrophage-colony stimulating factor (M-CSF) gene show impaired fertility (96). In addition, female mice that lack a functional leukemia inhibitory

factor (LIF) gene possess a functional blastocyst that fails to implant, and as a result, the fetus does not develop (97).

Wegmann and co-workers proposed that the maintenance of a successful pregnancy can be attributed to the Th2-like or anti-inflammatory environment that exists in the uterus during pregnancy. They showed that the murine feto-placental unit constitutively secretes detectable levels of IL-4, IL-5, and IL-10 throughout gestation (98, 99). Others have shown that cytokines associated with Th1 cells or proinflammatory cytokines, such as IL-2, IL-12, TNF-α, and IFN-γ, are not detectable at the fetal-maternal interface (100, 101). According to Mosmann, the Th2 subset of cytokines that includes IL-4, IL-5, IL-6, IL-10, and IL-13 are associated with a strong antibody mediated response and are considered helpful to pregnancy (102). In contrast, elevated Th1 cytokines, including TNF-α, IFN-γ, IL-2, IL-12 and lymphotoxin, are associated with a strong cell-mediated immune response and are considered to be harmful to pregnancy (103, 104). Consistent with Wegmann's proposal, autoimmune diseases associated with antibody-mediated immunity, such as systemic lupus erythematosus (SLE), worsen during pregnancy, and those associated with cell-mediated immunity, such as multiple sclerosis, uveitis, and rheumatoid arthritis, improve during pregnancy (105, 106). In addition, during pregnancy, the incidence of malignancies is more frequent and skin grafts survive for a prolonged period of time due to altered cell mediated immunity (107, 108). Infectious diseases, such as toxoplasmosis, leprosy, and malaria require elevated cell mediated immunity for their resolution, and as a

result are exacerbated during pregnancy (99). A mouse model of *Leishmania* infection demonstrates that in the case of a life-threatening infection, the pregnancy is sacrificed and a cell-mediated immune response is elicited to fight the infection (109). In summary, exacerbation of infections that require a cell-mediated immune response and evidence of anti-inflammatory cytokine production during pregnancy strongly suggests that a shift towards a Th2 or anti-inflammatory response is crucial to preventing feto-placental damage and suppressing cell-mediated immune responses.

Interleukin 10 (IL-10) is an anti-inflammatory cytokine expressed by T cells, macrophages/monocytes, B cells, mast cells, and keratinocytes (110). This pleiotropic cytokine has a molecular weight of 18 kDa, consists of 4 α-helical bundles, and tends to form noncovalent homodimers (111, 112). Similar to other cytokines, IL-10 is thought to induce it's biological activity by binding to receptors on the cell surface resulting in the association of two or more receptors (113). IL-10 was originally described as a product secreted by murine Th2 cell clones that inhibited cytokine synthesis by Th1 cell clones, activated macrophages, and natural killer cells (114, 115). IL-10 has many functions; including acting as a mast cell and T cell growth factor (116); promoting B cell differentiation and proliferation into antibody-producing cells (117); upregulating MHC class II expression on the surface of resting B cells (118); enhancing surface expression of FcγR (119); and suppressing many macrophage activities. IL-10 down regulates the production of IL-1α, IL-1β, IFN-γ, TNF-α, IL-3, IL-6, IL-8, GM-CSF, G-CSF,

reactive nitrogen and oxygen intermediates that are critical to elimination of intracellular and extracellular parasites. IL-10 also down regulates the expression of MHC Class II molecules, costimulatory molecule B7, and adhesion molecule ICAM-1 (120-125).

IL-10 seems to be crucial in preventing feto-placental damage and in suppressing the cell-mediated immune response. The importance of IL-10 in rodent pregnancy has been clearly demonstrated in the CBA X DBA/2 mating combination, a mouse model of immune pregnancy loss (126, 127). Treatment of pregnant CBA females with recombinant IL-10 caused increased fetal survival, while treatment with anti-IL-10 antibodies resulted in decreased fetal survival in this model system (128). During human pregnancy, the cytotrophoblast layer of the placenta and peripheral blood lymphocytes express IL-10 (129, 130). Clearly, there is a correlation between the presence of IL-10 at the fetal-maternal interface and success of pregnancy.

Placental, uterine, decidual, and immune cells secrete the cytokines and inflammatory mediators necessary to generate the shift in the immune response observed during pregnancy (131). As mentioned previously, this environment is crucial in preventing rejection of the semi-allogeneic fetus by the maternal immune system. Functional B and T cells do not constitute a significant cell population present in the murine decidua (132). Macrophages, and a subset of lymphocyte natural killer (NK) cells are present in the cycling and pregnant uterus of mice, rats, and humans (131). During mammalian pregnancy, macrophages

rapidly increase in density compared to other uterine cells present at the maternal-fetal interface (133-135). In humans, there is a substantial increase in the number of circulating monocytes and uterine macrophages, and this is observed in all three trimesters of pregnancy (136, 137). Hormones, such as progesterone, and cytokines, such as TGF-β and CSF-1, are produced by the cytotrophoblast and may act as chemoattractants for macrophages (138, 139). In rodents, macrophages cluster at the implantation site and are later redistributed throughout the myometrium, endometrial stroma, and metrial gland (140-142). In humans, the connective tissue and mesenchymal stroma of the pregnant uterus and mesenchymal region of the placental villi are rich in macrophages (141, 142).

The functions of uterine macrophages include: assisting in tissue remodeling that occurs during trophoblast invasion, defending against microbial invasion, and secreting immunosuppressive molecules (140, 143, 144). Activated macrophages have been shown to aid in the generation of an immunosuppressive uterine environment by synthesizing prostaglandin E_2 (PGE_2) and preventing the influx of maternal anti-fetal cytotoxic lymphocytes (24, 143, 145). The hormones, progesterone and estrogen have been shown to modulate macrophage function and to induce the production of anti-inflammatory cytokines by T cells (146-148). Other immuno-modulators, such as TGF-β and CSF-1, have also been reported to regulate macrophage function (149-153). In addition, murine macrophages have been reported to inhibit lymphocyte proliferation (143). It is likely that an

immunosuppressive phenotype in uterine macrophages would be advantageous to the semi-allogeneic fetus.

Large granulated lymphocytes (LGLs) are also found in the pregnant uteri of rats, mice, and humans (154). These cells arise from bone marrow LGL precursors. As rodent pregnancy progresses, the LGL marker (LGL-1) is lost and the cells develop mature granulated metrial gland (GMG) cell markers, such as Thy-1, NK1.1, and asialo-GM_1, that are common to natural killer cells (155, 156). Linnemeyer et al. showed that GMG cells, also known as uterine NK cells, are not derived from the T cell lineage and are not classical NK cells (157). In mice, the uterine NK cells localize to a small region of the implantation site called the mesometrial triangle, forming a specialized structure known as the granulated metrial gland (158, 159). Following implantation, uterine NK cells are redistributed to the primary decidua and the labyrinthine region of the placenta. Human LGLs are primarily abundant during the first trimester, while the murine counterparts are present throughout gestation (160).

The uterine NK cells function to eliminate microbes, and aberrant cells (141). In addition, they are suggested to control invasion of the trophoblast and destroy fetal cells that express Class I MHC molecules (161, 162). In a mouse model of fetal loss, elevated levels of uterine NK cells at the site of implantation are associated with an increased fetal resorption rate (163). The upregulation of anti-inflammatory cytokines at the maternal-fetal interface prevents the activation of NK cells and the subsequent destruction of fetal tissue by lymphocyte activated

NK (LAK) cells (164). In addition, the presence of HLA-G on the surface of human trophoblast cells may bind to uterine NK cells, inhibiting their activation and providing protection from the subsequent NK cell mediated lysis (91).

Pregnancy Failure

In humans, approximately 30% of all implanted embryos fail to develop to term (165). Fetal death may result from abnormalities in the ovum-zygote, uterine defects (166), or maternal factors such as infections (167), endocrine disorders (168, 169), and progesterone deficiency (170). However approximately 50% of all failed pregnancies are suggested to have an immunological etiology (171). Pregnancy loss and perinatal complications often result from disruption of the balanced immune environment that exists at the maternal-fetal interface (172, 173).

Recurrent spontaneous abortion (RSA), the most common complication of human pregnancy, is defined as three or more consecutive spontaneous abortions that occur before the 20^{th} week of gestation (174). In humans, defective production of IL-10, leukemia inhibitory factor (LIF), and interleukin-4 (IL-4) by decidual T cells has been associated with unexplained recurrent abortions (53). Reports indicate that macrophages and peripheral blood mononuclear cells from women with a history of recurrent spontaneous abortions are stimulated *in vitro* by sperm and trophoblast antigens to secrete IFN-γ and TNF-β (171, 175, 176). In addition, elevated numbers of NK and LAK cells were found to be associated with

elevated circulating levels of IL-1β, TNF-α, and IFN-γ (163). These cytokines may affect the fetus directly or indirectly through their effects on placental trophoblast cells or by leading to vasculitis (177, 178). TNF-α and IFN-γ stimulate expression of procoagulant (fgl2-prothrombinase) on the surface of maternal uterine vascular endothelium. Clotting is initiated by fgl2, and the resulting ischemic damage impedes maternal blood supply to the embryo (179).

There are two murine models of spontaneous abortion that have been studied extensively (180). The first model system, *Muc caroli-Mus musculus*, is a xenogeneic system (181). Since xenopregnancy is rarely successful and not the normal mode of reproduction, a second model of immune pregnancy loss, the CBA X DBA/2 mating combination, was developed (127, 182). This mating combination has a high frequency of spontaneous abortions (182). Mating of CBA/J females with DBA/2 males results in resorption of approximately 50% of the litter, while normal mating with BALB/c males result in less than 7% resorptions (127, 183, 184). BALB/c and DBA/2 males both possess the same H-2 haplotype (183, 185). There is substantial evidence that the resorption rate in the CBA/J mice is immunologically mediated. First, uterine NK cells infiltrate the implantation site prior to onset of fetal resorption (163). Stimulating the maternal immune system with lymphocytes possessing paternal alloantigens can significantly reduce the resorption rate (127, 185, 186). In addition, prevention of fetal resorption can be adoptively transferred to virgin CBA/J females by T cells from CBA/J females that were previously immunized with BALB/c cells (185).

The mechanism of embryo loss is not clearly understood. However, resorption in the CBA X DBA model system is dependent on the presence of high levels of TNF-α, IFN-γ, and IL-12, proinflammatory cytokines that are known to be detrimental to pregnancy, at the fetoplacental interface (163, 187-189). Enhanced fetal death is mediated by uterine NK cells that infiltrate and destroy the semi-allogeneic fetus (161, 162, 164). Administration of TNF-α and IFN-γ causes an increase in the already elevated resorption rate. Conversely, treatment with neutralizing antibodies to TNF-α results in a decrease in the resorption rate to almost normal levels (190). Agents such as lipopolysaccharide (LPS), a component of the cell wall of Gram negative bacteria, are known to induce pregnancy loss in normal rodent and human pregnancies (191). LPS stimulates the production of IL-1β, TNF-α, and IL-12 by macrophages and uterine NK cells present in uteroplacental tissue (142, 183, 186, 187, 192, 193). Riviera and co-workers recently reported that treatment of rats with a low dose of endotoxin (100 μg/kg, i.p.) resulted in 43% fetal loss and reduced size of the remaining concepti. Elevated levels of TNF-α were detected in the uterine tissue of these animals (194). In addition, LPS synergizes with TNF-α and IFN-γ to induce fetal resorption in mice (195). Reports indicate that upregulation of COX-2, an inducible isoform of cyclooxygenase, in the murine decidua is a crucial mediator of LPS-induced fetal death (196). The effect of LPS on the outcome of pregnancy

may be amplified by IFN treatment that functions to further activate macrophages (197).

Treatment of pregnant CBA females with recombinant IL-10 caused an increase in fetal survival in these animals. Conversely, treatment with anti-IL-10 antibodies on days 6, 8, and 10 of gestation results in an increased resorption rate (128). When CBA females are mated with BALB/c males, the placenta secretes IL-10 and the pregnancy progresses normally (185). The data in the CBA/J X DBA/2 mating combination strongly support the concept that a shift in the immune response occurs during pregnancy. Fetal survival is associated with reduced delayed-type hypersensitivity and NK cell reaction, and elevated maternal antibody responses (humoral immunity). In addition, the association of IL-10 and fetal survival suggests that IL-10 may aid in the generation of the anti-inflammatory uterine environment (128).

Given the existing data, it appears that agents regulating cytokine expression during pregnancy may play a critical role in the maintenance of the semi-allogeneic fetus. Because PSGs are present at such high levels during pregnancy and have been shown to bind to macrophages, it is reasonable to hypothesize that they may aid in the development of the unique immune environment that exists during pregnancy. A murine model was utilized to address whether PSGs modify the expression of cytokines and immune mediators by macrophages.

II. MATERIALS AND METHODS

Materials:

Animals

Five to six-week-old C3H/HeJ and BALB/c female mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and NCI (Frederick, MD), respectively. The animals were housed in cages with filter tops and fed standard laboratory chow and water ad libitum.

Cells

Peritoneal exudate macrophages were obtained from C3H/HeJ and BALB/c mice by peritoneal lavage with sterile saline four days after intraperitoneal injection of 3 ml of sterile 3% fluid thioglycollate broth (Difco, Detroit, MI). Cells were washed and resuspended in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Quality Biological, Inc., Gaithersburg, MD), 10mM HEPES (Irvine Scientific, Santa Anna, CA), 0.3% sodium bicarbonate, and 2% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah). Cells were added to 24-well tissue culture plates at 1.0×10^6 or 1.5×10^6 cells per well. After an 18 hour incubation, the nonadherent cells were removed by washing with medium. This procedure routinely yields >98% of macrophages (198). RAW 264.7 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in DMEM with high glucose (Irvine Scientific, Santa Anna, CA) supplemented with 10% FBS and 0.25 μg/ml Amphotericin B, 100 U/ml penicillin and 100 μg/ml streptomycin (Quality

Biologicals, Inc.), and 10 mM glutamine (Quality Biological, Inc.) as specified by ATCC.

The insect cells used for virus propagation and protein production were *Sf9* and *Hi5* cells (Invitrogen, Carlsbad, CA), respectively. The *Sf9* cells were grown in Sf900 II media (Life Technologies, Gaithersburg, MD) in the presence of 5% fetal bovine serum and the *Hi5* cells were grown in ExCell 400 media (JRH Biosciences, Lenexa, KS) under serum-free condition. Both cell types were maintained as specified by their manufacturer.

Reagents

Protein-free *Escherichia coli* K235 lipopolysaccharide (LPS) was obtained by hot phenol-water extraction, and was kindly provided by Dr. Stefanie Vogel (USUHS, Bethesda, MD).

The radioactive compound, $\gamma[^{32}P]ATP$ was bought from ICN and used in the 5' end-labelling procedures as described in the methods section.

The enzymes used in the experiments presented in this dissertation were obtained from various manufacturers. Thermostable DNA polymerase, cloned from *Thermus aqauticus*, was purchased from both Perkin Elmer and Sigma Chemical and Vent DNA polymerase was purchased from New England Biolabs. PFU DNA polymerase was obtained from Stratagene. For DNA ligation Ready-To-GoT4 DNA Ligase was acquired from Pharmacia Biotech. The Reverse Transcription reactions, described in the methods section, utilized Ready-To-Go You-First Strand Beads that were obtained from Amersham-Pharmacia Biotech.

The reverse transcriptase, Superscript RT from Life Technologies (Gaithersburg, MD) was utilized to clone the 3' end of Psg18 cDNA. The T4 Polynucleotide Kinase as well as most restriction enzymes used were from New England Biolabs. All of the enzymes were used according the to manufacturers' protocols. For sequencing, the Taq DNA polymerase was supplied in the Taq Dyedeoxy Terminator Cycle Sequence Kit (Applied Biosystems, Foster City, CA).

The vector BlueScript KS+ and the PCRscript cloning kit were purchased from Stratagene. The expression vectors pcDNA myc His 3.1A and pFastBAC1 were obtained from Life Technologies. Subcloning efficiency competent *E. coli* cells (DH5α) and Bacmid containing competent *E. coli* cells (DH10BAC) were from Life Technologies. Luria Bertani (LB) broth that was used for bacterial cultures was acquired from Sigma Chemicals. The agar plates were generated with LB Agar (Difco) and either 50 μg/ml ampicillin (Sigma, St. Louis, MO), or 50 μg/ml kanamycin, 10 μg/ml tetracycline, and 7 μg/ml gentamycin for DH5α or DH10Bac cells, respectively. Positive selection of inserted DNA was demonstrated by lack of complementation of the lac Z gene in plates prepared with Bluo-gal (Life Technologies) and IPTG. The Qiafilter plasmid protocol and reagents from Qiagen were used to prepare ultrapure plasmid DNA.

All agarose gels were cast at the required percentage (0.8-2%) using SeaKem GTG agarose (FMC). Acrylamide gels, purchased from Novex, were used for electrophoresis of proteins followed by coomassie staining or immuno blotting.

The 10% and 12% preparative acrylamide gels were generated with Acrylamide/Bis, TEMED, and ammonium persulfate (APS) provided by BioRad. The nytran and nitrocellulose membranes that were utilized for Southern and Western blot analysis and colony screening were obtained from Schleicher and Schuell (Keene, NH). The Microcon 3 and 100 and the Centriprep 3, 10, and 30, purchased from Millipore, were used for concentration of solutions.

The Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA) was used for sequencing of cDNAs cloned into plasmid vectors, according to the manufacturer's instructions. The TRIzol Reagent acquired from BRL was utilized to extract RNA from RAW 264.7 cells and C3H/HeJ macrophages. RNA was isolated from placental tissue using the RNAqueous protocol obtained from Ambion (Austen, TX).

The oligonucleotides were synthesized with an Applied Biosystems DNA Synthesizer (Applied Biosystems, Inc.) at the USUHS core facility and all generated had at least 95% purity. The sequences of all sense and antisense primers used for RT-PCR were selected from different exons and are shown in Table 1. The annealing temperatures used in the PCR reactions, the washing temperatures for the oligonucleotide probes, and the expected size of each PCR product are also shown in Table 1. The temperature shown in Table 1 was utilized for the wash with 5X SSPE/0.1% SDS. The sequences of all other oligonucleotides are referred to by number and are described in Table 2.

TABLE 1. Oligonucleotide sequences used for inflammatory mediator/cytokine RT-PCR. The oligonucleotides are listed in 5'→3' orientation. The annealing temperatures used in the PCR reactions, washing temperatures for the probes and the expected size of the product are shown for each cytokine/inflammatory mediator, as well as for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). AS = antisense

IL-1β

Annealing temp.: 53° C	**Sense**	GGGATGATGATGATAACCTG
Washing temp.: 60° C	**AS**	TTGTCGTTGCTTGGTTCTCCT
Size of product: 195 bp	**Probe**	CAGCTGCACTACAGGCTCCG

IL-6

Annealing temp.: 51° C	**Sense**	TTCCATCCAGTTGCCTTCTTG
Washing temp.: 50° C	**AS**	CTTCATGTACTCCAGGTAG
Size of product: 359 bp	**Probe**	ACTTCACAAGTCCGGAGA

IL-10

Annealing temp.: 46° C	**Sense**	CGGGAAGACAATAACTG
Washing temp.: 55° C	**AS**	CATTTCCGATAAGGCTTGG
Size of product: 190 bp	**Probe**	GGACTGCCTTCAGCCAGGTGAAG-ACTTT

IL-12p40

Annealing temp.: 57° C	**Sense**	CGTGCTCATGGCTGGTGCAAA
Washing temp.: 50° C	**AS**	GAACACATGCCCACTTGCTG
Size of product: 575 bp	**Probe**	GCTCAGGATCGCTATTAC

TNF-α

Annealing temp.: 54° C	**Sense**	TTCTGTCTACTGAACTTC
Washing temp.: 57° C	**AS**	CTCCAGCTGGAAGACTCCTCCCAG
Size of product: 436 bp	**Probe**	GGGATGAGAAGTCCCCAAATG

TGF-β

Annealing temp.: 60° C	**Sense**	CTCCCCACTCCCGTGGCTTCTAG
Washing temp.: 51° C	**AS**	GTTCCACATGTTGCTCCACACACTTG
Size of product: 472 bp	**Probe**	CCGGGAGGCCAGCCG

iNOS

Annealing temp.: 64°C	**Sense**	CCCTTCCGAAGTTTCTGGCAGCAGC
Washing temp.: 60° C	**AS**	GGCTGTCAGAGCCTCGTGGCTTTGG
Size of product: 497 bp	**Probe**	CAAGGTCTACGTTCAGGACATC

GAPDH

Annealing temp.: 50° C	**Sense**	CCATGGAGAAGGCTGGGG
Washing temp.: 53° C	**AS**	CAAAGTTGTCATGGATGACC
Size of product: 195 bp	**Probe**	CTAAGCATGTGGTGGTGCA

TABLE 2. Oligonucleotides used to clone murine *Psg* cDNAs. The oligonucleotides are categorized according to their purpose, in a 5'→3' orientation. Nucleotides in parentheses represent positions of degeneracy within the oligonucleotide. The introduction of restriction enzyme recognition sequences is designated by nucleotides that are bolded. For all sequences given, the underlined nucleotides indicates additional sequences that are not homologous to the cDNA. The washing temperature is provided for any oligonucleotide utilized as a probe. AS = antisense

Cloning Cea6 Leader

5758 (Cea61s)	Sense	AAGGAAGGACAGCAAAT
5759 (Cea61AS)	AS	AGCTGTGAGCAGAAGAC
6042 (JWA) wash at 60° C	Probe	GAGGTGTCCTTGGTGCTTCTC

Cloning full length Psg18

5932 (Cea31S)	Sense	CTCCTCTCTTTTCATCTGT
5930 (CONAS)	AS	TCACTCATTT(AG)TCACAGCCAG
5934 (Cea33S) wash at 55° C	Probe	CTGGTACAAAGGGCTGATTG

Cloning full length Psg19

5861 (AccS)	Sense	CGTCGACGGAGAAAATGTTC
5859 (AccAS)	AS	GAACATTTCTCCGTCGAC
8050 (4AS.2)	Sense	GCTCTAGA (*Xba* I) GAGAAGAGATATGGA-GGTGTCCTCTGAGCTTCTCAGCAA-TGGGTGG ACCTCCTGGCAGAGGG-TTCTGCTCACAGCCTCCCTCTT
5863 (4ASS)	Sense	GCTCTAGA (*Xba* I) GAGAAGAGATATG
5865 (4AAS)	AS	TTTCTCCGTCGAC (*Acc* I) CAATTTGGGTGGTA-CGGA TTCGATGGTGACTCGGGCAGTGA-TCGGC AGGAACCAGCAGGTTAAGAGGG

5864 (4AASS)	AS	TTTCTCCGTCGACCAATTGG *(Acc I)*

Sequencing of Psg18 – 3'end

4404 (T7s)	Sense	GTAATACGACTCACTATAGGGC
2051 (M13rev)	AS	AACAGCTATGACCATG
6845 (CEA34S)	Sense	GTGCTTAGTACTCAGGACTTT
6846 (CEA3AS1)	AS	TGGACTTCGTTGCTATGCTAT

Cloning Psg17, 18, and 19 into pAcSecG2T

5963 (Bgl2)	Sense	GAAGATCTAGAGTCACTGTGGAATT (*Bgl* II) (AA 1 of PSG17 N1-domain)
5961 (CEA2ST)	AS	TCACACGATCATCACAGCCAG
5964 (Bgl34)	Sense	GAAGATCTCGAGTCACCAT(TC)GAATC (*Bgl* II) (AA 1 of PSG18 & 19 N1-domains)
5962 (CEA4ST)	AS	TCACACATTCATCACAGCAG
5930 (CONAS)	AS	TCACTCATTT(AG)TCACAGCCAG

Verify that Psgs are in frame

6167 (GP67GST)	Sense	ATGTGCCTGGATGCGTTCCA

Cloning GST-Psg18N

8959 (C3S)	Sense	GGGAATTCATGCTACTAGTAAATCAGTC (*Eco* RI) (AA 1 of GP67 Leader)

8960 (C3AS1)	AS	AGATGCACGACGGGAGTACACGTG-CAAGTAC
8961 (C3AS2)	AS	CCGGTACCTAAACAGATGCACGACGGG (*Kpn* I)

Cloning Psg18N-His

6984 (BGLMAS)	Sense	GAAGATCTAGAGATATGGAG(TG)TGTC (*Bgl* II) (AA 1 of PSG leader)
11683 (3NKS)	AS	TCAGTACCGGAGTACACGTGCAAG (Stop) (*Kpn* I)

Determining Sites of Psg Expression

7113 (M5' Uni)	Sense	TCTTCTCCAGGTCCATAA
7114 (M3' Uni)	AS	ATTG(GA)TACTCTCCAGCAT
7142 (C2) wash at 61° C	Probe	ACACAGCCCGTCATAAAAGCC
7140 (C3FL) wash at 55° C	Probe	GTACAAAGGTGTGCTTAGTAC
7307 (CPr) wash at 55° C	Probe	ATACAGCATAGTGACAGAGTC

Methods:

Preparation of plasmid DNA

Colony hybridization, using end-labelled oligonucleotide probes, was utilized to verify the presence of the desired insert. Small scale preparations of plasmid DNA (minipreps) were performed by the boiling lysis method (adapted from Holmes and Quigley, 1981). The minipreps typically yield an adequate amount of DNA for anaylsis by restriction enzyme digestion. After the plasmid was demonstrated to have the appropriate restriction enzyme digestion pattern, the *E. coli* strain (DH5α) colony that contained the recombinant plasmid was grown for 17 hours at 37° C in 25 ml of Luria bertani (LB) broth with 50ug/ml of ampicillin (Sigma). The maxiprep protocol from Qiagen was utilized to obtain ultrapure plasmid DNA.

Oligonucleotide labeling

For end-labelling oligonucleotides, approximately 200 ng of the appropriate oligonucleotide was incubated in the presence of $\gamma[^{32}P]ATP$, 10 units T4 polynucleotide kinase and 1X polynucleotide kinase buffer (1X buffer consists of 70 mM Tris-HCl [pH 7.6], 10mM $MgCl_2$, 5mM dithiothreitol) for 1 hour at 37° C (NEB, Beverly, MA). Following the one hour incubation, 25 µl dH_2O was added to the original 25 µl reaction, and the entire sample volume was passed through a G-25 column to remove unincorporated $\gamma[^{32}P]ATP$. The gamma counter was used to determine the number of cpm per µl of probe. The labelled oligonucleotide was

used at the concentration of 1 X 10^6 cpm/ml of hybridization solution. The remaining labeled oligonucleotide was stored at –20° C for approximately one week, and then discarded according to Environmental Health and Occupational Safety (EHOS) regulations.

Southern blotting

Amplified products were electrophoresed on 1-1.5% agarose gels and transferred to Nytran membranes (Scheicher & Schuell) overnight using 20X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaPO4, 1 mM EDTA [pH 7.7] and 0.1% SDS). The next morning, DNA was cross-linked with UV light for 2 minutes and baked onto the Nytran membrane in a vacuum oven for 30 minutes. Following 20-30 minutes of prehybrization with Rapid-hyb (Amersham Pharmacia Biotech, Piscataway, NJ), the membrane was hybridized with an internal ^{32}P-labeled oligonucleotide probe for a minimum of 1 hour. The washing procedure for all Southern blots was two 20 minute room temperature washes with 1X SSPE/0.1% SDS followed by two 20 minute washes at high temperature [T_m - 5° C] with 5X SSPE/0.1% SDS. Depending on the purpose of the Southern blot, the signal was either visualized on an autoradiograph or quantitated using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cloning of the full length *Psg18* cDNA

To obtain the 3' end of the *Psg18* cDNA, total RNA was extracted from 18-day placentas of Swiss Webster mice using the TRIzol reagent. Three micrograms of the RNA sample were reverse transcribed with Superscript RT (Life

Technologies, Gaithersburg, MD) and a degenerate consensus antisense primer (5930), designed based on the cDNA sequences of murine *Psg* family members *Psg17*, *Psg19*, and *Cea 6* (Rudert, 1992), according to the manufacturer's recommendations. The obtained cDNA was then amplified for 30 cycles [94°-1min. 30sec., 50°-1min. 30 sec., 72°-2 min.] using *Vent* DNA polymerase (New England Biolabs) with the same consensus antisense primer used for the reverse transcriptase reaction and a *Psg18*-specific sense primer (5932) that was designed based on the partial Psg18 5'sequence.

The PCR product was first cloned into pCRScript (Statagene, La Jolla, CA). The entire *Psg18* cDNA coding sequence was obtained by ligating the PCR product (encoding amino acid 143 to the stop codon) to partial *Psg18* cDNA (corresponding to the first 208 amino acids of Psg18) using a common *SstI* restriction site. Then, both strands were sequenced using the oligonucleotide prmers 4404, 2051, 8960, and 8961.

Cloning of Full length *Psg19*

A partial *Psg19* cDNA sequence was provided by Dr. Wolfgang Zimmerman. To generate the missing 5'end of the *Psg19* cDNA, a unique restriction site was introduced into the partial cDNA sequence by site-directed mutagenesis. A sense primer containing a single mismatched base (A → C), 5861, and a consensus antisense, 5930, were used to create an *Acc* I site (GTCGAC) between nucleotides 34 and 39 of the partial *Psg19* sequence (199). Then, a two part PCR reaction was performed to obtain the 5' sequence comprising the leader

peptide and the first 16 amino acids of the N1-domain. Overlapping primers, a sense 95mer (8050) and an antisense 81mer (5865), were designed based on the *Psg19* genomic sequence. These megaprimers were annealed to each other through their overlapping 3' ends at 48° C. The DNA fragment obtained was used as a template and amplified under standard PCR condtions with primers (5863 and 5864), designed to be complimentary to the 5' and 3'ends of *Psg19*. The sense primer contained a *Xba* I site at it's 5' end and the antisense primer included an *Acc* I site at it's 3' end. The obtained product was subcloned into pCRScript, excised from the vector with *Xba* I and *Acc* I and ligated to partial *Psg19* cDNA at the *AccI* site, resulting in the full length *Psg19* cDNA sequence.

Determination of tissue-specific expression

Eleven different adult mouse tissues from Swiss Webster mice, including liver, brain, thymus, heart, spleen, kidney, lung, testes, ovary, embryo, and 18 day placenta, were analyzed for expression of *Psg17*, *18*, and *19* mRNA by RT-PCR (200). RNA for all tissues, with the exception of the 18 day placenta, was obtained from Ambion (Austin, TX). The 18 day placental RNA was extracted from placental tissue (Pel-Freeze, Rogers, AR) using the RNAqueous protocol (Ambion) according to the manufacturer's specifications. RNA preparations were quantified by UV spectrophotometry and RT-PCR was performed in two separate steps. For each tissue, 2 μg of RNA was reverse transcribed using 0.2 μg of random hexamer primers and the Ready-To-Go You Prime First Strand Beads (Pharmacia Biotech, Piscataway, NJ) in a 33 μl reaction. Then, 3 μl of the cDNA

was amplified with "Universal" mouse primers to detect murine *Psg17*, *18*, and *19*. The M5' Universal primer (7113) hybridized to the N3-domain, while the M3' Universal primer (7114) hybridized to the A-domain. These primers were designed to be located in different exons, ensuring that the product was a result of cDNA amplification. The housekeeping gene (GAPDH) was amplified for each sample to monitor RNA integrity (200).

The amplified products were analyzed using standard Southern blotting techniques (201). Differential hybridization of Southern blots was performed with oligonucleotide probes 7142, 7140, or 7307 that were designed to be specific for *Psg17*, *18*, or *19*, respectively. Specificity was verified by testing for hybridization to linearized *Psg17*, *18*, and *19* cDNA clones, prior to use in this experiment. The oligonucleotide probes were end-labelled with $\gamma[^{32}P]$ATP as described previously, and the hybridization signals were detected by autoradiography.

Characterization of the *Cea6* cDNA clone

A fragment that included the beginning of leader sequence of *Cea6* was generated using the sense primer (5758) and the antisense primer (5759) which were designed to be complimentary to part of the 5'-untranslated region and the leader peptide, respectively. Four hundred nanograms of genomic DNA was extracted from 4 inbred mice strains (A/J, C57BL/6J, YBR/Ei and SWR/J) (Jackson Laboratory) and amplified for 30 cycles (94°-30 sec., 50°-30 sec., 72°-30 sec. for 30 cycles) with PFU DNA polymerase (Stratagene) following

manufacturer's instructions. The obtained products were passed through a Microcon 100 to remove unicorporated primers and dNTPs and cloned into pCRScript. Colony hybridization using the $\gamma[^{32}P]$ATP end-labeled 6042 probe was performed to identify the colony containing the desired product. The cloned products from all four mouse strains were sequenced using the Dye Terminator Cycle Sequencing Reaction Ready Mix (ABI Prism, Foster City, CA) as specifed by the manufacturer.

Generation of Recombinant Baculovirus constructs

To generate recombinant full length PSG17, 18, and 19 baculovirus constructs, PCR was utilized to amplify the respective cDNA sequences encoding the mature proteins. These constructs will possess a gp67 leader sequence, and not the *Psg* leader. All oligonucleotides were designed based on the known Psg cDNA sequences. *Psg17* cDNA was provided by Dr. Wolfgang Zimmerman (University of Freiburg, Germany) as a full length cDNA clone and amplified with sense primer, 5963, and antisense primer, 5961. The *Psg18* and *Psg19* cDNA sequence were both amplified with the same sense oligonucleotide primer, 5934, but with two different antisense primers. The *Psg18* was amplified with the consensus antisense primer, 5930, while *Psg19* was amplified with the antisense primer, 5962. All of the antisense oligonucleotides consisted of the complimentary 3' *Psg* sequence ending with the stop codon. The sense primers (5961 and 5963) used to amplify *Psg17*, *18* and *19* both contained a *Bgl* II recognition sequence at their 5' end, followed by the respective Psg sequence

beginning with first amino acid of the mature protein. Therefore, the products amplified from all 3 murine *Psgs* contained a *Bgl* II site at their 5' end and a 3' blunt terminus. The 1.4-1.6 kb *Psg17*, *18* and *19* PCR products were digested with *Bgl* II and ligated to the baculovirus expression vector, pAcSecG2T (PharMingen, San Diego, CA), downstream from the polyhedron promoter, gp67 leader, and GST-tag. Prior to cloning, the baculovirus vector was previously digested with *Bam* HI and *Sma* I. The resulting constructs were referred to as pAcSecG2T-*Psg17*, *18*, or *19*.

To generate the GST fusion protein containing only the N1-domain of PSG18 (GST-PSG18N), the pAcSecG2T-*Psg18* construct was used as the template. Since it was necessary to add more than 30 nucleotides to the 3' sequence, the amplification was done in two parts. First, linearized pAcSecG2T-*Psg18* was amplified with the sense primer, 8959, and the antisense primer, 8960. The product obtained from this reaction was used at the template for the second amplification with the same sense primer, 8959, and antisense primer, 8961. The sense oligonucleotide consisted of a 5' *Eco* RI recognition sequence followed by the sequence of the first seven amino acids of the gp67 leader peptide. The first antisense primer contains the 3' sequence to the N1-domain followed by the beginning of the kinase site obtained from the Ek/LIC vector (Novagen, Madison, WI), while the second antisense primer consisted of the remainder of the kinase site and *Kpn* I restriction site (202). Amplification with these oligonucleotides generated a product that contained *Eco* RI and *Kpn* I restriction sites at it's 5' and

3' ends, respectively. The product consisted of the gp67 leader, and the GST tag followed by the N1-domain of *Psg18* sequence. Removal of unincorporated primers and nucleotide was accomplished by passing the amplified material through a Microcon 100 microconcentrator (Amicon). The sample was recovered in a 20 μl volume and cloned into pFastBAC1 (Life Technologies). Prior to ligation, the pFastBAC1 vector was digested with *Eco* RI and *Kpn* I, then recovered from a 0.8 % agarose gel by centrifugation of the agarose gel piece and ethanol precipitated.

PSG18N was also generated as a 6x Histidine fusion protein. To generate the PSG18N-His, the pAcSecG2T-*Psg18* plasmid was linearized and amplified with the upstream primer, 6984, and the downstream primer, 11683. The sense oligonucleotide contained a *Bgl* II site, followed by 6 nucleotides that were derived from the 5'-untranslated region, and the first 4 amino acids of the leader peptide of the *Psg18* cDNA. The antisense oligonucleotide included the last 16 nucleotides of the *Psg18* N1-domain, followed by a *Kpn* I restriction site and a stop codon. To add the 6x Histidine tag, the PCR product was cloned into the *Bam* HI-*Kpn* I sites of pcDNA3.1(-)/Myc-His A (Invitrogen). This construct was referred to as *Psg18N-* pcDNA3.1(-)/Myc-His A. Prior to ligation, the amplified product and the pcDNA3.1(-)/Myc-His A vector were digested with *Bam* HI and *Kpn* I, recovered from a 1 % agarose gel by centrifugation and ethanol precipitated. Then, *Psg18N*-His was excised from pcDNA3.1(-)/Myc-His A with *Pme* I and ligated to the pFastBAC1 that was digested with *Stu* I. The orientation

of the insert was verified by restriction enzyme analysis. In addition, the presence of the N1-domain sequence was confirmed by colony hybridization with an internal probe (5934) that is specific to a region within the N1-domain of *Psg18* (203). The *Psg18N*-His in pFastBAC1 consists of the leader sequence and the N1-domain of *Psg18*, followed by a Myc and 6x His tag.

Production of Recombinant Baculovirus Stocks Expressing GST-PSG17, GST-PSG18, GST-PSG19, GST-PSG18N, PSG18N-His and GST-His XylE

Recombinant virus expressing GST-PSG17, GST-PSG18, and GST-PSG19 were obtained by co-transfection of the plasmid pAcSEcG2T-*Psg17*, *18*, or *19*, respectively, with BaculoGold DNA, a linearized wild type genomic viral DNA, into *Sf9* cells. The progeny virus was plaque-purified following the manufacturer's instructions (PharMingen, Los Angeles, CA). The recombinant virus was identified by an altered plaque morphology (occlusion negative), characterized by the absence of occluded virus in the nucleus of the infected *Sf9* cells. Plaques that contained recombinant GST-PSG17, 18, or 19 virus were picked and the virus isolated. Then, the virus was propagated in *Sf9* cells to generate high titer (P4) virus stocks for the production of soluble GST-PSG17, GST-PSG18, and PSG19. The recombinant glycoproteins were secreted into the supernatant of infected *Hi5* or *Sf9* cells.

The recombinant virus expressing the control fusion protein GST-His XylE was obtained by co-transfection of BaculoGold DNA and the baculovirus transfer vector pAcGHLT-XylE (Pharmingen) into *Sf9* insect cells as indicated above for

GST-PSG17, 18, and 19. Insect cells infected with the recombinant GST-His-XylE baculovirus resulted in the production of a non-glycosylated cytoplasmic protein.

Recombinant baculoviruses expressing GST-PSG18N or PSG18N-His were obtained using the protocol provided by Life Technologies. The DH10Bac competent *E. coli* cells, containing the bacmid with a mini-attTN7 target site and the helper plasmid, were transformed with pFastBAC-GST-Psg18N or pFastBAC-Psg18N-His plasmid DNA. The Qiagen protocol was used to obtain ultrapure recombinant plasmid DNA for the transformation. The mini-Tn7 element on the pFastBAC1 plasmid was transposed to the mini-attTn7 target site on the bacmid in the presence of transposition proteins, supplied by the helper plasmid. White colonies containing the recombinant bacmids were identified by disruption of the lacZ α gene. *E. coli* colonies containing the transposed DNA were selected and high molecular weight miniprep DNA was prepared. This high molecular weight DNA from Psg18N-His or GST-Psg18N was transfected into *Sf9* cells with CellFECTIN reagent (Life Technologies). The virus was collected 3 days later and referred to as the recombinant P1 virus stock. The virus was propagated as described above for the full length GST-PSG17, 18, and 19 and recombinant protein was produced.

Production and purification of recombinant proteins

GST-PSG18N and PSG18N-His fusion proteins were produced and purified from culture supernatants. *Hi5* cells were infected at a multiplicity of infection

(MOI) of 1, then grown in ExCell 400 serum-free media (JRH Biosciences) for 72 hours at 27° C. GST-PSG17, 18, and 19 were produced in *Hi5* cells supplemented with 5% fetal bovine serum. The culture supernatants collected at 72 hours were maintained in the presence of 1% phenylmethsulfonyl flouride (PMSF) and 1% leupeptin (Sigma). All supernatants were clarified by centrifugation and concentrated approximately 5-10 fold using an ultrafiltration cell model 8400 with a YM 10K filter (Amicon), a Centriprep 3, or 10 (Amicon). Supernatants that contained the full length PSGs were concentrated 50-100 fold prior to use experiments.

As for the other recombinant fusion protein described above, *Hi5* or *Sf9* cells maintained under serum free conditions were infected at an MOI of 5 with the GST-His-Xyl E virus stock and incubated for 72 hours at 27° C. The GST-His XylE remained in the cytoplasm. To obtain the recombinant protein, the insect cells were lysed with RIPA buffer (0.1M NaCl, 0.001 M EDTA [pH7.4], 0.1% Nonidet P-40, 0.1% deoxycholate, 1% phenylmethysulfonylfluoride, and 1% aprotinin) for 45 min on ice. Cellular debris was removed by centrifugation at 2000 x g for 5 minutes. The clarified cell lysate that contained the recombinant GST-His XylE was incubated from 2 hours to overnight at 4°C with glutathione-sepharose beads (Pharmacia Biotech). Then, the glutathione-Sepharose beads were washed and 10 mM glutathione was used to elute the recombinant protein. The eluted protein was concentrated with a Centriprep 10.

To purify the recombinant GST-PSG18N and PSG18N-His, 5 ml of the concentrated material, as described above, were loaded on a 10% or 12% SDS-PAGE gel respectively in the model 491 Prep Cell apparatus (BioRad, Hercules, CA) which separates proteins by size. Fractions containing the recombinant proteins of interest were identified by immunoblot analysis and pooled. The SDS was removed by electroelution in 10 mM Tris buffer, pH 8.0. For this purpose, the Sialomed apparatus (Amika Corp., Columbia, MD) was used with dialysis membranes that have a molecular weight cutoff of 10 kDa (Amika Corp., Columbia, MD). After SDS removal, recombinant GST-PSG18N and PSG18N-His were further concentrated with a Centriprep 10 or Centriprep 3, respectively.

The final concentrations of the recombinant proteins were determined by comparison to bovine serum albumin (BSA) standards using the Eagle Eye II Still Video System and Eagle Sight version 3.1 software (Stratagene, La Jolla, CA). The proteins were separated on 4-20% NuPAGE SDS-polyacrylamide gels (Novex, San Diego, CA) and stained with Gel Code Blue (Pierce, Rockford, IL). The concentration of GST-PSG18 was determined by a different method, using PSG18N as a standard. GST-PSG18 was compared to known concentrations of GST-PSG18N after imunoblotting with the anti-GST antibody followed by goat-horseradish peroxidase conjugated anti-mouse antibody. In order to stay within the linear range of detection of the Super Signal system (Pierce), different exposures of the autoradiograph were analyzed in the Eagle Eye system.

Semi-quantitative cytokine and inflammatory mediator RT-PCR (204)

TRIzol reagent (Life Technologies) was used according to the manufacturer's protocol to extract total cellular RNA from macrophages and RAW 264.7 cells. The RNA pellet was resuspended in 20-30 μl of sterile water and quantified by UV spectrophotometry. To generate cDNA, RT-PCR was performed in two separate steps. First, 2-4 μg of RNA were reverse transcribed using the Ready To Go U Prime First Strand beads (Pharmacia Biotech, Piscataway, NJ) and 0.2 μg of random hexamers in a 33 μl reaction. Next, the PCR amplifications were performed using 1.5-3 μl of cDNA as the template, 40 pmoles of each sense and antisense primer, 1mM each of dATP, dCTP, dTTP, and dGTP, 1X PCR buffer I (10X buffer contains 500 mM KCl, 100 mM Tris-HCl [pH 8.3], 15 mM $MgCl_2$, and 0.01 % w/v gelatin), and 2.5 U Ampli*Taq* DNA polymerase (Perkin-Elmer, Foster City, CA) in a 50 μl reaction. Primers sequences, with the exception of the TGF-β primer set, have been published previously (200, 204-207). The sequences of the oligonucleotides used for amplification and detection of TGF-β were generated in the USUHS synthesizing facility and are shown in Table 1.

PCR amplification cycle number and RNA input were varied over a broad range for each pair of primers to establish conditions that resulted in a linear correlation between RNA input and PCR product, allowing for comparison of the relative amounts of the immune mediator of interest and GAPDH mRNA. The

cDNAs were amplified 14, 17, 27, 23, 22, 17, 20, and 28 cycles for GAPDH, IL-1β, IL-10, IL-12p40, TNF-α, iNOS, and TGF-β, respectively. All PCR amplifications were performed at least three times with multiple sets of experimental RNAs. After Southern blottting as described above, the data sets were individually normalized for the relative quantity of mRNA by comparison to GAPDH. Fold induction was determined by comparing the normalized values of PSG treated samples to the control-treated samples.

Generation of polyclonal antibody to PSG18N (pAbPSG18N)

Large quantities of GST-PSG18N obtained from infected *Hi5* cell supernatant were concentrated 10 fold using a Centriprep 10 (Amicon). The concentrated material was separated on a 6 % SDS polyacylamide preparative gel and stained with 0.5 % Coomassie Brilliant Blue R-250 (BioRad) in dH_2O. The band on the gel that corresponded to the 45 kDa GST-Psg 18N was excised and cut into small pieces using a sterile razor blade. The fragments of acrylamide were used to immunize 2 rabbits. Pre-immune, first, second, and third bleeds were collected from the animals. Using immunoblots, the bleeds were tested for reactivity to PSG18N. Once reactivity was confirmed, the third bleed was purified using a protein G sepharose column (Pharmacia). Then, the third bleed was passed through a GST column (Pierce) to remove potential antibodies to the GST-tag present in the rabbit sera. The reactivity of purified pAbPSG18N was tested on an immunoblot of PSG18N and full length PSG18 and 19.

Concanavalin A sepharose precipitation

One mililiter of medium collected from insect cells that were infected with recombinant GST-PSG18 or GST-PSG18N baculoviruses was mixed with 100 µl of Concavalin A-sepharose 4B (Sigma) in Con A buffer (0.05M sodium phosphate buffer pH 7.0, containing 0.2 M NaCl and 0.02% sodium azide) and incubated overnight at 4°C. The beads were spun down at 1,300xg and washed three times with Con A buffer. All glycoproteins bound to the Con A sepharose were eluted with 0.5M α-D-methylmannoside in Con A buffer.

Immunoblot analysis

The recombinant proteins and prestained molecular weight standards were separated on SDS-PAGE (4-12% gels), transferred to nitrocellulose membrane (Scheicher & Schuell), and blocked overnight in TBST (25 mM Tris [pH7.6], 150 mM NaCl, 0.1% Tween 20) supplemented with 5% powdered milk at 4° C (208). The membranes were then incubated with 1µg/ml of anti-GST mAb (Pharmingen) in TBST-milk for 1 hour. After three 5 minute washes, the membranes were incubated for 1 hour with a 1:3,000 dilution of goat-horseradish peroxidase conjugated anti-mouse antibody (BioRad). For detection of PSG18N-His protein, the membranes were blocked for 1 hour in TBST supplemented with 2.5 mg/ml of BSA, followed by a 2 hour incubation with the INDIA HisProbe-HRP (Pierce). The last step in this protocol is the same for all immunoblots. Following three, 20 minute washes the membranes were incubated for 5 minutes with the Super Signal

chemiluminescent substrate (Pierce) and the bound horseradish peroxidase was detected by autoradiography.

IL-10 and IL-6 ELISA

For detection of the cytokines, IL-6 and IL-10, 1 x 10^6 thioglycollate-elicited BALB/c peritoneal macrophages and RAW 264.7 cells were treated for 4 hours at 37° C with the indicated concentrations of GST-PSG18, GST-PSG18N, PSG18 His or the control protein, GST-His XylE, in the presence or absence of LPS. The RAW 264.7 cells were treated with 1 ng/ml of LPS, while the peritoneal macrophages were treated with 100 ng/ml of LPS. All treatments were performed in a total volume of 300 µl. After the initial 4 hour treatment, 700 µl of media were added to each well and the cells were incubated for an additional 16-20 hours at 37° C. The culture supernatants were harvested after a 20-24 hours post-treatment.

The media collected from the macrophages was concentrated three-fold using a Microcon 3, while the media collected from RAW 264.7 cells was diluted 3 fold prior to measuring IL-10 by enzyme-linked immunosorbent assay (ELISA) (Endogen, Woburn, MA) according to manufacturer's specifications. The IL-6 ELISA (R&D Systems, Minneapolis, MN) was performed on the same samples. All LPS-treated samples were assayed undiluted, and diluted 1:3 and/or 1:6 in media to assure the amount of IL-6 detected would be within the linear range of the standard curve. Supernatants that were not exposed to LPS were not diluted

prior to the assay. The lower limits of detection were 25 and 37 pg/ml for total IL-6 and IL-10 protein, respectively.

Analysis of endotoxin contamination

Preparations of the GST-PSG18, GST-PSG18N, GST-His-XylE and PSG18N-His were assayed for their level of endotoxin contamination. The *Limulus* amebocyte lysate assay was performed by Charles River Endosafe (Charleston, SC). Protein samples were only used if endotoxin levels were less than 0.2 ng/ml.

Statistical analysis

Data were analyzed using the Sigma Plot statistical package (Jandel Scientific software). Comparisons between two groups were made using the unpaired *t* test. Data were considered statistically significant if *p* values were 0.05 or less. All experiments were repeated at least three times with similar results.

III. RESULTS

Cloning and characterization of murine pregnancy specific glycoproteins

To date, only four murine cDNAs derived from the fourteen known *Psg* partial gene sequences have been cloned. These are: murine *Cea6*, *Psg17*, *Psg18*, and *Psg19*. The full length *Psg 17*, as well as the partial *Psg18* and *Psg19* cDNAs, were provided by Dr. Wolfgang Zimmerman (University of Freiburg, Germany). B. Kromer and co-workers published the partial 5'sequence of *Psg18*, but the 3'sequence from amino acid 143 to the stop codon was not known. It has been previously published that murine *Psgs* are specifically expressed by the placenta (209). Therefore, placental RNA was used to obtain the full length *Psg18* cDNA. Using a sense primer based on the previously published 5' *Psg18* sequence and a consensus antisense primer derived from 3' end of *Psg17*, *19* and *Cea6* sequences, a 1 kb product encoding the 3' sequence of Psg18 was generated. The full length *Psg18* sequence was generated by ligating the PCR product to the partial cDNA provided by Dr. Zimmerman and was then sequenced. The full length nucleotide, as well as the deduced amino acid sequence of *Psg18* are depicted in Figure 1. Similar to other murine PSGs, PSG18 consists of a 34 amino acid leader peptide, followed by three immunoglobulin (Ig) variable-like or N-domains and one immunoglobulin constant-like or A-domain. The GenBank accession number for the *Psg18* cDNA sequence is AF 128236.

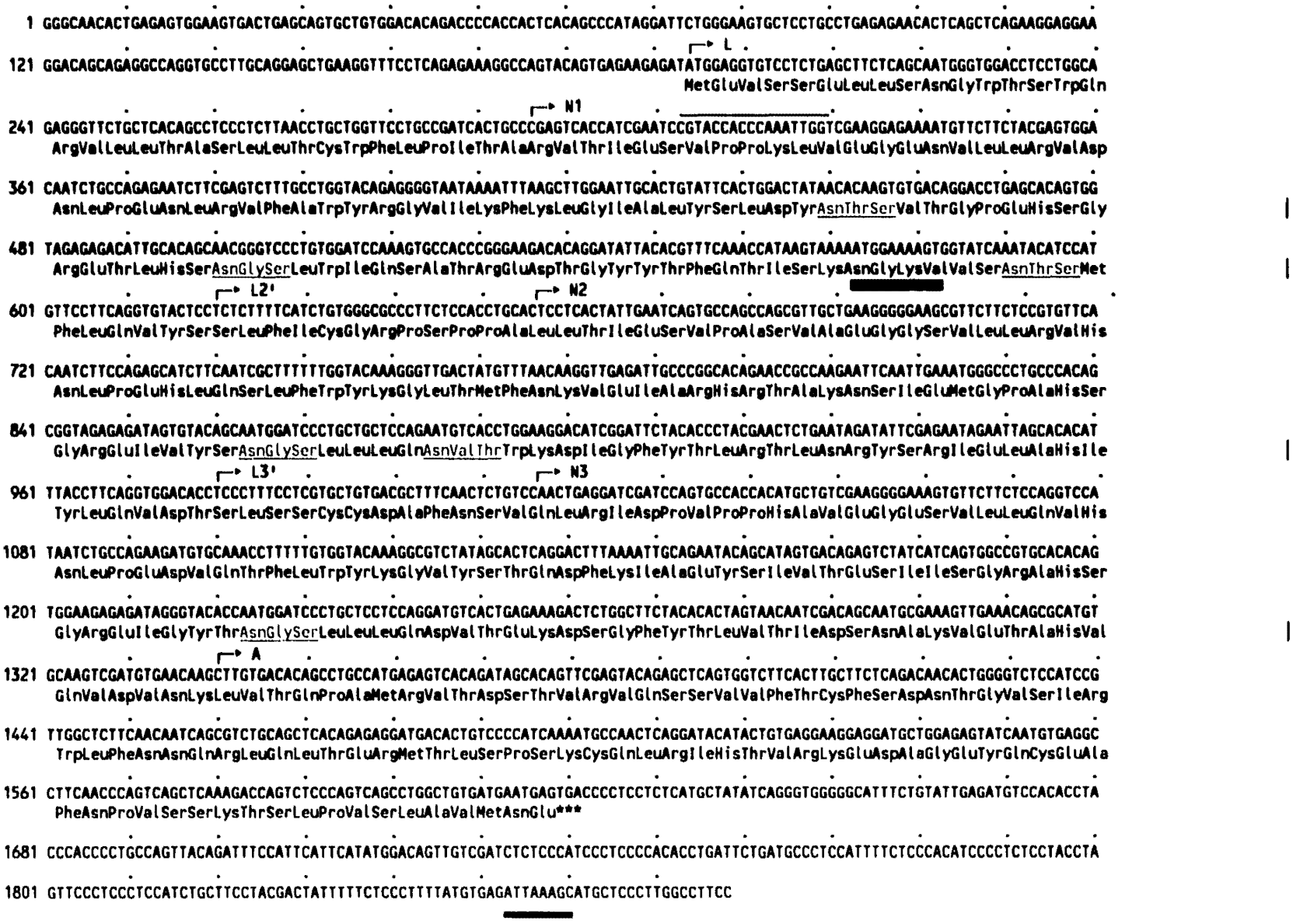
Figure 1. Nucleotide and derived amino acid sequence of *Psg18* (accession number AF 128236). The nucleotide numbers are shown in the left margin. The start sites of the leader (L), the Ig variable-like (N) domains and the Ig constant-like (A) domain are indicated by arrows. The N2 and N3 domains are preceded by a 12 amino acid leader sequence which are designated L2' and L3', respectively. Potential N-linked glycosylation sites are thinly underlined. As depicted, *Psg18* has 7 potential glycosylation sites. The stop codon is represented by 3 asterisks in the amino acid sequence.


L
N1
L2'
N2
L3'
N3
A

For *Psg19*, the nucleotide sequence encoding the leader peptide and the beginning of the N-terminal domain were missing from the cDNA obtained previously. Using site-directed mutagenesis (199), an *Acc* I site was generated within the N1-domain of *Psg19*. Overlapping DNA fragments, based on the genomic sequence of *Psg19*, and primers designed based on the flanking sequences of these fragments were used to amplify the sequence of the leader and the first 16 amino acids of the N-terminal domain. The full length sequence shown in Figure 2 was generated by ligating the 165 bp PCR product to the partial cDNA clone using the common *Acc I* site.

Rudert and coworkers cloned the *Cea6* cDNA from a BALB/c placental cDNA library. The sequence of the isolated *Cea6* showed a single base deletion that corresponds to the tenth amino acid of the leader peptide, resulting in a frame-shift (35). To verify this finding and determine whether *Cea6* is a pseudogene in BALB/c as well as other inbred mouse strains, primer were designed to amplify a 146 bp region of the *Cea6* gene which includes the 5' untranslated region and the first 50 nucleotides of the leader sequence. The PCR products obtained from the DNA of the 4 mouse strains examined (A/J, C57BL/6, YBR/Ei, and SWR/J) were sequenced and compared to that of the *Cea6* cDNA isolated from BALB/c mice. Nucleotide 30 was deleted in the genomic DNA of all 4 inbred mouse strains examined. These data strongly suggest that *Cea6* is a pseudogene, therefore no further work was done with this cDNA.

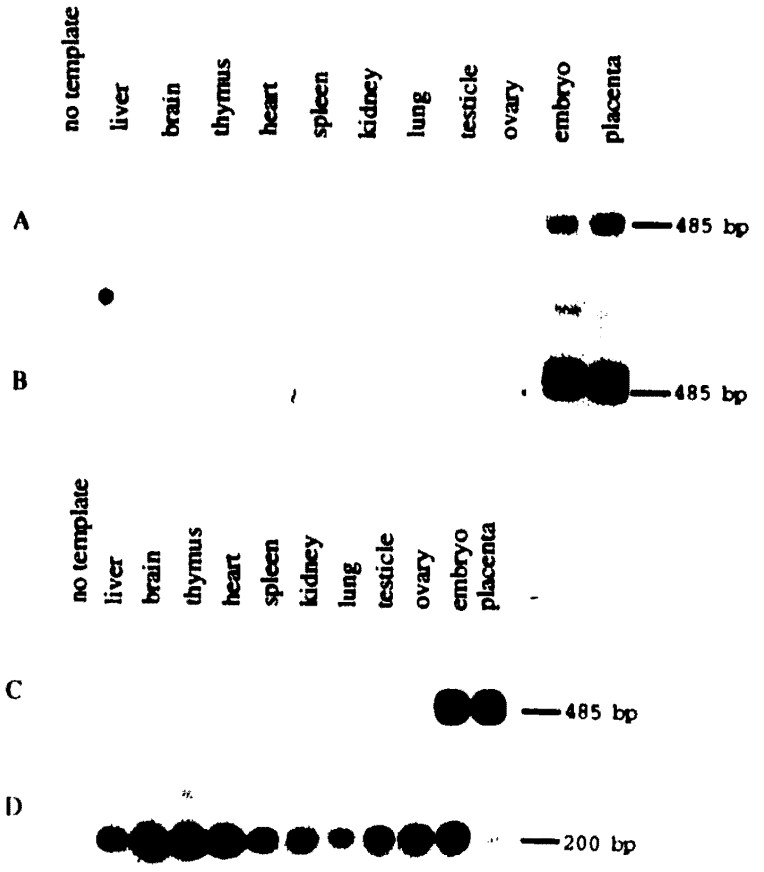
Figure 2. Nucleotide and derived amino acid sequence of *Psg19*. The nucleotides are numbered on the left margin. The start sites of the leader (L), the 3 Ig variable-like (N) domains, separated by 12 amino acid leader-like sequence (L'2, L'3), and the Ig constant-like (A) domain are indicated by arrows. The putative N-linked gycosylation sites are typed in gray and thinly underlined. *Psg19* has 6 potential gylcosylation sites. The stop codon is designated by 3 asterisks in the amino acid sequence. The polyadenylation signal is indicated by a thick line in the last line of nucleotides. The thin line over a portion of the N1-domain represents the start of the missing cDNA sequence generated by PCR to produce to full length *Psg19* cDNA sequence.


1 GGGCAACACTGAGAGTGGAAGTGACTGAGCAGTGCTGTGGACACAGACCCCACCACTCACAGCCCATAGGATTCTGGGAAGTGCTCCTGCCTGAGAGAACACTCAGCTCAGAAGGAGGAA
L
121 GGACAGCAGAGGCCAGGTGCCTTGCAGGAGCTGAAGGTTTCCTCAGAGAAAGGCCAGTACAGTGAGAAGAGATATGGAGGTGTCCTCTGAGCTTCTCAGCAATGGGTGGACCTCCTGGCA
MetGluValSerSerGluLeuLeuSerAsnGlyTrpThrSerTrpGln
N1
241 GAGGGTTCTGCTCACAGCCTCCCTCTTAACCTGCTGGTTCCTGCCGATCACTGCCCGAGTCACCATCGAATCCGTACCACCCAAATTGGTCGAAGGAGAAAATGTTCTTCTACGAGTGGA
ArgValLeuLeuThrAlaSerLeuLeuThrCysTrpPheLeuProIleThrAlaArgValThrIleGluSerValProProLysLeuValGluGlyGluAsnValLeuLeuArgValAsp
361 CAATCTGCCAGAGAATCTTCGAGTCTTTGCCTGGTACAGAGGGGTAATAAAATTTAAGCTTGGAATTGCACTGTATTCACTGGACTATAACACAAGTGTGACAGGACCTGAGCACAGTGG
AsnLeuProGluAsnLeuArgValPheAlaTrpTyrArgGlyValIleLysPheLysLeuGlyIleAlaLeuTyrSerLeuAspTyrAsnThrSerValThrGlyProGluHisSerGly
481 TAGAGAGACATTGCACAGCAACGGGTCCCTGTGGATCCAAAGTGCCACCCGGGAAGACACAGGATATTACACGTTTCAAACCATAAGTAAAAATGGAAAAGTGGTATCAAATACATCCAT
ArgGluThrLeuHisSerAsnGlySerLeuTrpIleGlnSerAlaThrArgGluAspThrGlyTyrTyrThrPheGlnThrIleSerLysAsnGlyLysValValSerAsnThrSerMet
L2'
N2
601 GTTCCTTCAGGTGTACTCCTCTCTTTTCATCTGTGGGCGCCCTTCTCCACCTGCACTCCTCACTATTGAATCAGTGCCAGCCAGCGTTGCTGAAGGGGGAAGCGTTCTTCTCCGTGTTCA
PheLeuGlnValTyrSerSerLeuPheIleCysGlyArgProSerProProAlaLeuLeuThrIleGluSerValProAlaSerValAlaGluGlyGlySerValLeuLeuArgValHis
721 CAATCTTCCAGAGCATCTTCAATCGCTTTTTTGGTACAAAGGGTTGACTATGTTTAACAAGGTTGAGATTGCCCGGCACAGAACCGCCAAGAATTCAATTGAAATGGGCCCTGCCCACAG
AsnLeuProGluHisLeuGlnSerLeuPheTrpTyrLysGlyLeuThrMetPheAsnLysValGluIleAlaArgHisArgThrAlaLysAsnSerIleGluMetGlyProAlaHisSer
841 CGGTAGAGAGATAGTGTACAGCAATGGATCCCTGCTGCTCCAGAATGTCACCTGGAAGGACATCGGATTCTACACCCTACGAACTCTGAATAGATATTCGAGAATAGAATTAGCACACAT
GlyArgGluIleValTyrSerAsnGlySerLeuLeuLeuGlnAsnValThrTrpLysAspIleGlyPheTyrThrLeuArgThrLeuAsnArgTyrSerArgIleGluLeuAlaHisIle
L3'
N3
961 TTACCTTCAGGTGGACACCTCCCTTTCCTCGTGCTGTGACGCTTTCAACTCTGTCCAACTGAGGATCGATCCAGTGCCACCACATGCTGTCGAAGGGGAAAGTGTTCTTCTCCAGGTCCA
TyrLeuGlnValAspThrSerLeuSerSerCysCysAspAlaPheAsnSerValGlnLeuArgIleAspProValProProHisAlaValGluGlyGluSerValLeuLeuGlnValHis
1081 TAATCTGCCAGAAGATGTGCAAACCTTTTTGTGGTACAAAGGCGTCTATAGCACTCAGGACTTTAAAATTGCAGAATACAGCATAGTGACAGAGTCTATCATCAGTGGCCGTGCACACAG
AsnLeuProGluAspValGlnThrPheLeuTrpTyrLysGlyValTyrSerThrGlnAspPheLysIleAlaGluTyrSerIleValThrGluSerIleIleSerGlyArgAlaHisSer
1201 TGGAAGAGAGATAGGGTACACCAATGGATCCCTGCTCCTCCAGGATGTCACTGAGAAAGACTCTGGCTTCTACACACTAGTAACAATCGACAGCAATGCGAAAGTTGAAACAGCGCATGT
GlyArgGluIleGlyTyrThrAsnGlySerLeuLeuLeuGlnAspValThrGluLysAspSerGlyPheTyrThrLeuValThrIleAspSerAsnAlaLysValGluThrAlaHisVal
A
1321 GCAAGTCGATGTGAACAAGCTTGTGACACAGCCTGCCATGAGAGTCACAGATAGCACAGTTCGAGTACAGAGCTCAGTGGTCTTCACTTGCTTCTCAGACAACACTGGGGTCTCCATCCG
GlnValAspValAsnLysLeuValThrGlnProAlaMetArgValThrAspSerThrValArgValGlnSerSerValValPheThrCysPheSerAspAsnThrGlyValSerIleArg
1441 TTGGCTCTTCAACAATCAGCGTCTGCAGCTCACAGAGAGGATGACACTGTCCCCATCAAAATGCCAACTCAGGATACATACTGTGAGGAAGGAGGATGCTGGAGAGTATCAATGTGAGGC
TrpLeuPheAsnAsnGlnArgLeuGlnLeuThrGluArgMetThrLeuSerProSerLysCysGlnLeuArgIleHisThrValArgLysGluAspAlaGlyGluTyrGlnCysGluAla
1561 CTTCAACCCAGTCAGCTCAAAGACCAGTCTCCCAGTCAGCCTGGCTGTGATGAATGAGTGACCCCTCCTCTCATGCTATATCAGGGTGGGGGCATTTCTGTATTGAGATGTCCACACCTA
PheAsnProValSerSerLysThrSerLeuProValSerLeuAlaValMetAsnGlu***
1681 CCCACCCCTGCCAGTTACAGATTTCCATTCATTCATATGGACAGTTGTCGATCTCTCCCATCCCTCCCCACACCTGATTCTGATGCCCTCCATTTTCTCCCACATCCCCTCTCCTACCTA
1801 GTTCCCTCCCTCCATCTGCTTCCTACGACTATTTTTCTCCCTTTTATGTGAGATTAAAGCATGCTCCCTTGGCCTTCC

Expression of Psgs in Embryo, Placenta, and Tissues of Non-Pregnant Mice

Expression of mRNA encoding human *PSGs* has been reported in some tissue of males and non-pregnant females (15, 16). Therefore, it was investigated whether expression of murine *Psgs* could be detected in tissues other than the placenta. For this purpose, RNA was obtained from adult murine liver, brain, thymus, heart, spleen, kidney, lung, ovary, embryo (mid-gestation), and 18 day placenta (as a positive control). Expression of *Psg17*, *18* and *19* mRNA was analyzed by RT-PCR. "Universal" primers were designed to amplify *Psg17*, *18* and *19* generated a 485 bp product. For all tissues, amplification of the housekeeping gene, GAPDH, produced the expected 200 bp product. Expression of the different *Psg* family members in the eleven tissues examined was determined by differential hybridization with specific oligonucleotide probes. As illustrated in Figure 3, expression of *Psg17*, *18*, and *19* was observed only in the embryo and placental tissues. The lower intensity GAPDH signal in the lane that corresponds to placental RNA, suggests that the quantity of *Psg* mRNA expressed in the placenta is much greater than the amount expressed by the embryo. In addition, *in situ* hybridization using embryonic tissue, showed no expression of *Psg18* mRNA (Finkenzeller, personal communication). Therefore, these results indicate that expression of Psgs is specific to the placenta.

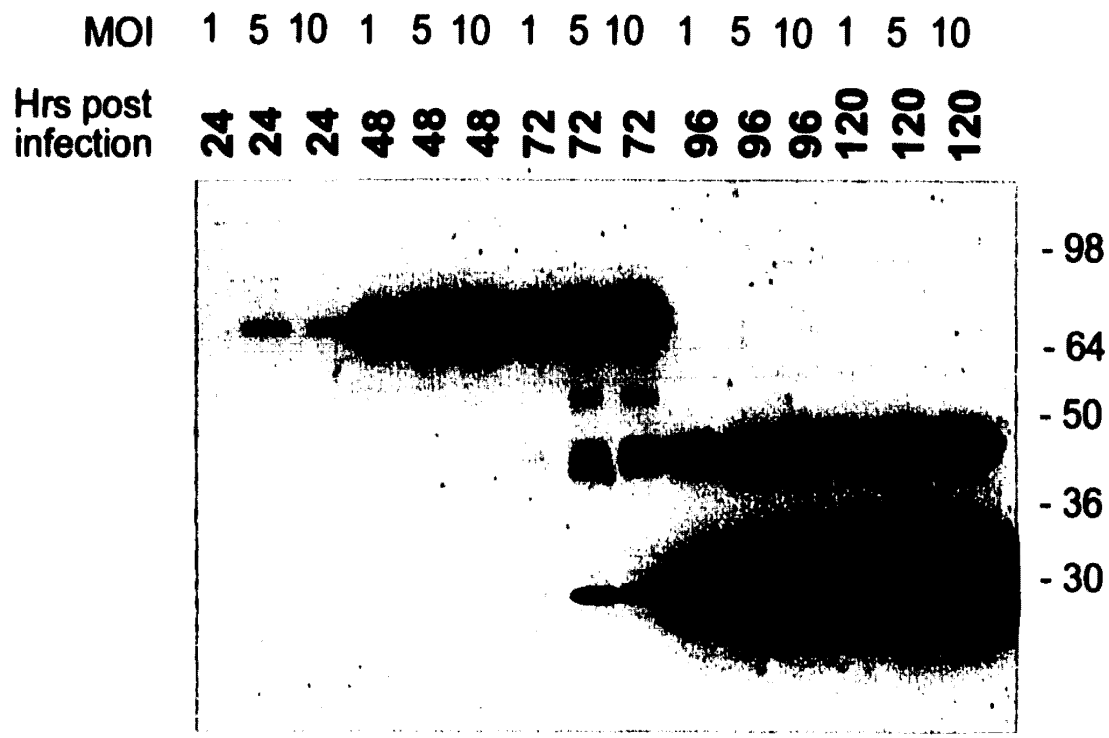
Figure 3. Detection of *Psg* mRNA in embryo, placenta, and tissues of non-pregnant adult mice by RT-PCR. Following reverse transcription of total RNA from the designated tissues, *Psg* cDNA fragments were amplified with the M5' universal (7113) and M3' universal (7114) primer set. These primers amplify *Psg17*, *Psg18*, *Psg19*, *Cea6*, and possibly additional murine *Psg* cDNAs that have not yet been cloned. Fifteen microliters of each PCR product was electrophoresed in triplicate on 1.5 % agarose gels. Using standard Southern blotting techniques the DNA to nylon membranes, and hybridized with ^{32}P-end-labeled oligonucleotides (7142, 7140, or 7307) specific for *Psg17* (A), *Psg18* (B), or *Psg19* (C), respectively. All three *Psg* probes hybridized to the 485 bp products generated from placental and embryo RNA, but not to the RNA obtained from the various non-pregnant mouse tissues. The additional product visualized with the *Psg18* probe (B) may represent an unknown splice variant. (D) Southern blot hybridization of the GAPDH PCR products, used as an internal control to monitor the RNA integrity of all RNA samples.


no template
liver
brain
thymus
heart
spleen
kidney
lung
testicle
ovary
embryo
placenta
A
485 bp
B
485 bp
no template
liver
brain
thymus
heart
spleen
kidney
lung
testicle
ovary
embryo
placenta
C
485 bp
D
200 bp

Production of recombinant murine Pregnancy Specific Glycoproteins

Baculovirus, an eukaryotic expression system, was used to generate the recombinant PSG17, PSG18, PSG19, and XylE proteins as glutathione-S-transferase (GST) fusion proteins. *Psg17*, *18*, and *19* cDNAs were cloned into the baculovirus expression vector, pAcSecG2T, and introduced into baculovirus behind the strong polyhedron promoter by homologous recombination. In this system, transfer vectors are engineered to contain genes that will undergo homologous recombination with the linearized *Autographica Californica* nuclear polyhedrosis virus (AcNPV) DNA when cotransfected into Sf9 cells. Insect cells infected with the recombinant viruses produced secreted proteins with an N-terminal GST-tag. For all proteins, a time course of infection was performed to determine the time required for optimal protein production. Figure 4 shows the time course performed for PSG18. Hi5 cells were infected with PSG18 P4 virus stock at a multiplicity of infection (MOI) of 1, 5, or 10 and supernatants were harvested 24, 48, 72, 96, or 120 hours post-infection. The anti-GST immunoblot shown in Fig. 4 is representative of the time course results obtained for all the full length PSGs. As depicted for PSG18, supernatant harvested 72 hours post-infection with virus at an MOI of 1 resulted in optimal protein production, with the least degradation products. Infection for longer times or with an MOI greater than 1 resulted primarily in the detection of smaller products that reacted with the anti-GST antibody. The smaller products had molecular weights of 30 and 45 kDa.

Figure 4. Time course of GST-PSG18 infection to determine the time point of optimal protein production. *Hi5* cells seeded at 1 x 10^6 in a 6 well plate were infected with GST-PSG18 P4 virus stock at a multiplicity of infection (MOI) of 1, 5, or 10. Cell supernatants were harvested at 24, 48, 72, 96, or 120 hours post-infection and electrophoresed on a 8-16% Tris-Glycine polyacrylamide gel under denaturing conditions. Recombinant PSG18 was detected by immunoblotting with anti-GST antibody (Pharmigen), followed by a HRP-conjugated goat anti-mouse antibody (BioRad), and Super Signal chemiluminescent substrate (Pierce). The 72 hour time point at an MOI of 1 appears to be optimal for expression of the 85 kDa, PSG18. Infection with higher MOIs or for longer time points results in the production of smaller proteins, possibly truncated forms of full length PSG18. The molecular weight standards shown to the right of the figure are in kilodaltons. The time course shown is representative of the results obtained for GST-PSG17, 19, and GST-His-XylE.


MOI 1 5 10 1 5 10 1 5 10 1 5 10 1 5 10
Hrs post infection 24 24 24 48 48 48 72 72 72 96 96 96 120 120 120
- 98
- 64
- 50
- 36
- 30

The XylE control is a *Pseudomonas putrida* gene that encodes a 40 kDa protein. When transfected with Baculogold DNA, the pAcGHLT-XylE control vector generates proteins with both a N-terminal GST and 6x His tag. As for the PSGs, a time course was performed for the XylE protein production (data not shown). Supernatant harvested at 72 hours post-infection with XylE P4 virus stock at an MOI of 5 was determined to be optimal for production of protein. Figure 5 shows the detection of specific fusion proteins. The full length GST-PSG17, 18, and 19 have molecular weights of 80-85 kDa, and the control GST-His-Xyl E has a molecular weight of 70 kDa. The molecular weight of the PSGs is indicative of some degree of glycoslyation. Psg 17, PSG18 and PSG19 consist of 463 amino acids with 6-7 potential N-linked glycosylation sites. To verify that the full length PSGs were glycosylated, the recombinant proteins were precipitated with concanavalin-A-sepharose. The Con A-Sepharose precipitated proteins are also depicted in Figure 5.

As previously mentioned, PSG17, 18, and 19 have a similar domain organization. Figure 6 is a comparison of the N1-domains of PSG17, 18, and 19. There is approximately a 55% amino acid identity between the N1-domain of PSG18 and that of other murine PSGs. As was observed for other members of the carcinoembryonic antigen (CEA) family, the N1-domain is likely to be critical to

Figure 5. Recombinant PSG17, 18, 19, and XylE fusion proteins produced in insect cells. *Hi5* insect cells were infected with PSG17, 18, 19, or XylE P4 virus stocks. Cell supernatants or lysates, containing the respective fusion proteins, were harvested 72 hours post-infection and loaded on a 4-20% NuPAGE gel. Recombinant proteins were detected by immunoblotting with an anti-GST antibody followed by HRP-conjugated goat anti-mouse antibody and the Super Signal detection reagent. GST-PSG17, 18, and19 before and after concanavalin A-sepharose precipitation (Con A ppt.) are depicted, indicating that the recombinant proteins are glycosylated. The full length PSGs and the GST-His-XylE control protein have molecular weights of 80-85 kDa and 70 kDa, respectively. A slightly smaller product is visualized in the lanes containing con-A-sepharose precipitated PSG18 and 19. The proteins are probably differentially processed, most likely due to different degrees of glycosylation. Sup = PSG Infected *Hi5* Cell Supernatant

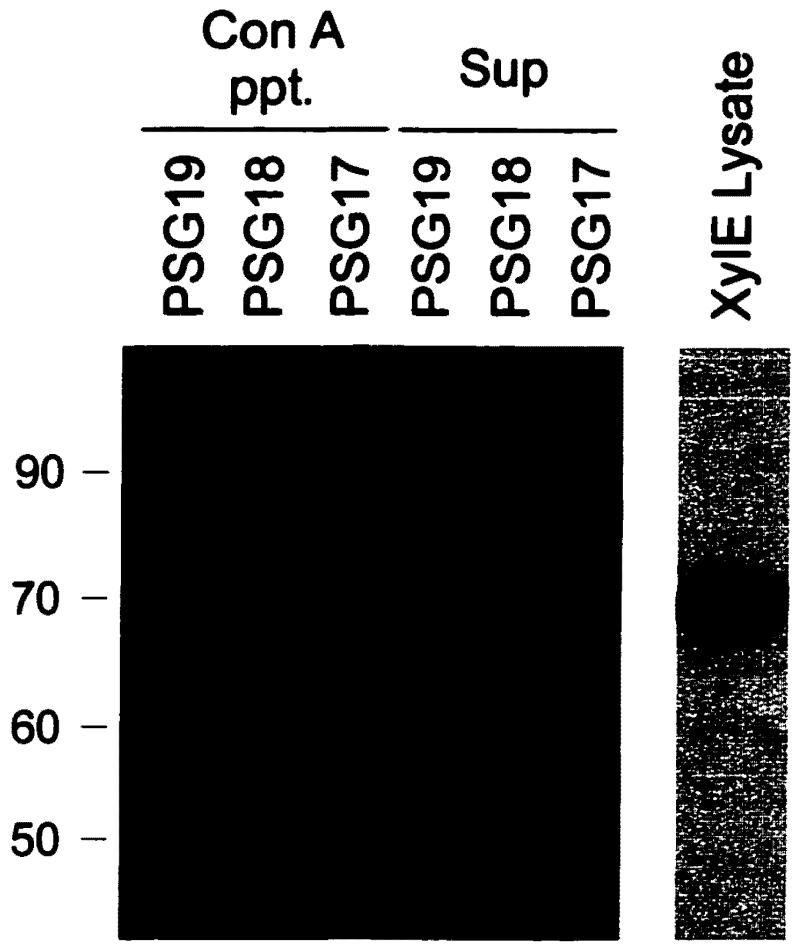

Con A ppt.
Sup
PSG19
PSG18
PSG17
PSG19
PSG18
PSG17
XylE Lysate
90
70
60
50

Figure 6. Comparison of the N1-domain of murine PSG17, 18, and 19. The sequence begins with amino acid 1 of the N1-domain and ends with amino acid 12 of the leader-like (L'2) domain, prior to the beginning of the N2-domain. The asterisks represent amino acids that are homologous to the sequence given for PSG17. The underlined sequence designates the location of the RGD-like motif. PSG19 does not possess an RGD-like sequence. All sequences were previously published by Zimmerman et al. (1992).

Comparison of Murine PSG N1 domains (108 AA)

PSG17 RVTVEFLPPQVVEGENVILLRVDNLPENLLGFVWYKGVAS

PSG18 ***I*S*****Y*****L******M*****V*G**R*MTN

PSG19 ***I*SV**KL**********RV*A**R**IK

PSG17 MKLGIALYSLQYNVSVTGLKHSGRETLHRNGSLWIQNVTS

PSG18 LWQA**QHW*Y*S*M*K**N*****I*YI**********Q

PSG19 F*********D**T****PE********S*******SA*R

PSG17 EDTGYYTLRTVSQRGELVSDTSIFLQVYSSLPICERPTTLV

PSG18 *******FQ*I*K***I**N**LY*H*****F**V*****I

PSG19 *******FQ*I*KN*KV**N**M********F**G**SPPA

protein function (210-213). In addition, there were numerous production and purification problems with the full length proteins that caused them to be an inadequate source of protein for functional studies. Therefore, a truncated version of PSG18 that contained only the N1-domain was generated with an N-terminal GST tag or a C-terminal 6x His tag. As determined by their size on anti-His or anti-GST immunoblots, PSG18N-His and GST-PSG18N have estimated molecular weights of 24 kDa and 45 kDa, respectively. The anti-GST and anti-His immunoblots of GST-PSG18N and PSG18N-His, respectively, are shown in Figure 7. The Concanavalin A-Sepharose precipitation of GST-PSG18N depicted on the anti-GST immunoblot in Figure 7. Concanavalin A binds molecules that contain α-D-glucopyranasyl and sterically related residues. GST-PSG18N binds to Con A, indicating that glycosylation occurs within the N1-domain of PSG18.

Supernatants containing either the GST-PSG18N or PSG18N-His fusion proteins were concentrated and separated on a SDS-polyacrylamide gel using the BioRad Prep Cell Model 491. The schematic shown in Figure 8 provides a detailed description of this purification strategy. Using the Prep Cell apparatus, glycoproteins with a molecular weight of 45kDa (GST-PSG18N) and 24 kDa (PSG18N-His) were purified to apparent homogeneity, based on the absence of other proteins after separation on a NuPAGE SDS-polyacrylamide gels (Novex) stained with Gel Code Blue (Pierce, Rockford, IL). Like the full length proteins, both of the recombinant PSG18N proteins generated in the baculovirus system

Figure 7. Recombinant GST-PSG18N and PSG18N-His produced in insect cells. *Hi5* insect cells were infected with the GST-PSG18N or PSG18N-His P4 virus stock. Cell supernatants containing the respective fusion proteins were harvested 72 hours post-infection. Twenty-five microliters of each supernantant was electrophoresed on a 4- 20 % NuPAGE gel and transferred to nitrocellulose. The recombinant proteins were detected by immunoblotting with an anti-GST or anti-His antibody, followed by goat HRP-conjugated anti-mouse antibody and the Supersignal chemiluminescent substrate. GST-PSG18N and PSG18N-His have an estimated molecular weight of 45 kDa and 23 kDa, respectively. Concanavalin-A-Sepharose precipitation of GST-PSG18N, indicates that glycosylation occurs within the N1-domain of PSG18.

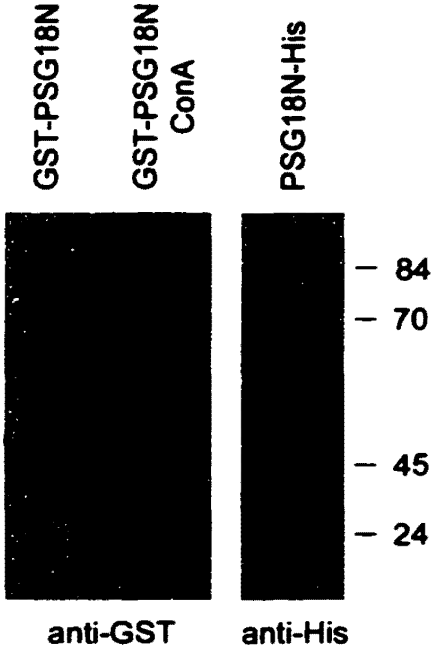

GST-PSG18N
GST-PSG18N ConA
PSG18N-His
84
70
45
24
anti-GST
anti-His

Figure 8. The purification of PSG18N. A schematic of the basic protocol used to generate homogeneous preparations of GST-PSG18N and PSG18N-His. This method of purification generated biologically active proteins that were used to study the functional role of PSG18.

Purification Scheme

Infect Hi5 Cells with P4 virus stock

Harvest supernatant at 72 hours

Concentrate supernatant ~5fold with a Centriprep 3 or 10

Run several SDS-polyacrylamide Prep Gels (5 ml/gel)

Collect 2.5 ml fractions

Run on SDS-PAGE to determine which fractions contain Psg 18N

Pool Fractions

Pass through Sialomed to remove SDS

Concentrate ~100 fold with Centriprep 3 or 10

Compare to BSA Standards on SDS-PAGE

Send for LAL Testing

Determine the Concentration

were not in an active conformation. The indicator of functional activity was induction of IL-10 mRNA in murine macrophages. Denaturation followed by the removal of SDS generated proteins with biological activity. Figure 9 depicts a Coomassie-stained polyacrylamide gel that illustrates the level of protein purity obtained using this means of purification.

Generation of Polyclonal antibody to PSG18N

Prior to beginning this study, there was no antibody available for the detection of murine PSGs. The available anti-human PSG antibodies showed no cross-reactivity with the murine proteins. This was expected, since the sequence of the human and mouse proteins is very different. Therefore, GST-PSG18N was utilized to immunize rabbits in an attempt to generate a polyclonal antibody specific to PSG18N. Pre-immune, as well as the 1st, 2nd, and 3rd bleeds of two rabbits (2090 and 2091) were tested for reactivity to GST-PSG18N and GST-His-XylE as a control. As expected the pre-immune sera did not react, while all three bleeds from both animals reacted with PSG18N and not GST-His-XylE. The sera from one of the rabbits (2090) reacted much stronger with Psg 18N. The 3rd bleed of this rabbit was purified with a protein G column, followed by a GST column to remove reactivity against the GST portion of the fusion protein. Then, specificity for PSG18, PSG18N and PSG19 was tested. As described above, Figure 10 shows that the anti-GST antibody and the pAbPSG18N both show specificity to the PSG 18N, while only the anti-GST antibody reacted with the GST-His-XylE. As shown in Figure 11, the anti-PSG18N polyclonal antibody demonstrated cross-

Figure 9. Purification of GST-PSG18N and PSG18N-His from insect cell supernatants. *Hi5* cells supernatants were collected 72 hours post-infection with GST-PSG18N or PSG18N-His P4 virus stocks and purified with the BioRad Prep Cell as described previously. The 4-20% NuPAGE gel stained with GelCode Blue, a type of coomassie staining, depicts both of the PSG18N fusion proteins before (lane 1) and after purification (lanes 2 and 3). The molecular weight standards (M) are in kilodaltons.

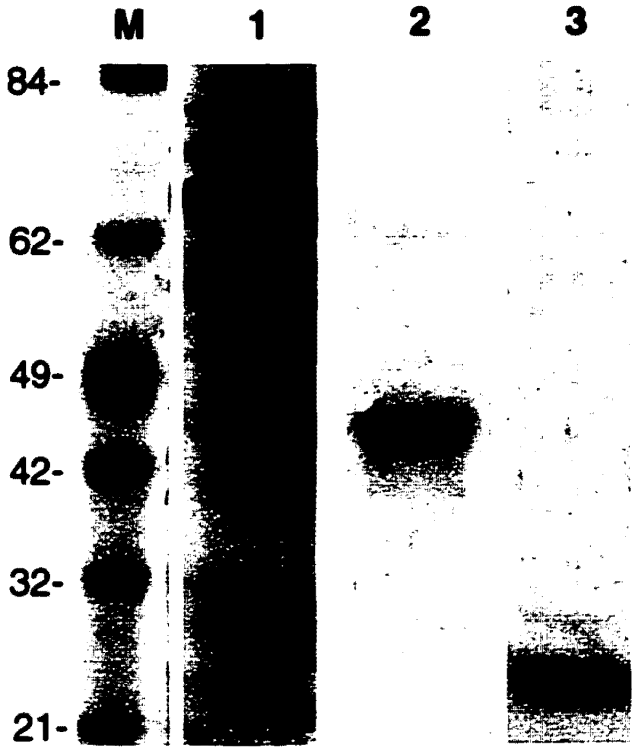

M
1
2
3
84-
62-
49-
42-
32-
21-

Figure 10. The polyclonal antibody, pAbPSG18N, reacts with PSG18N. *Hi5* supernatants containing either GST-PSG18N or GST-His-XylE were electrophoresed on 4-20 % NuPAGE gels in triplicate and transferred to nitrocellulose. The immunoblots were incubated with anti-GST, anti-PSG18N (pAbPSG18N), or pre-immune sera, followed by goat HRP-conjugated anti-mouse, or anti-rabbit antibody. After incubating the membranes with Super Signal chemiluminescent substrate, reactivity with the respective antibodies was detected by autoradiography. Although the autoradiograph of pAbPSG18N immunoblot shows a high degree of non-specific binding, it reacts specifically with the 45 kDa, GST-PSG18N. The pre-immune sera showed no reactivity with either the PSG18N or the XylE, while the anti-GST antibody reacted with both of the GST fusion proteins. The molecular weight standards shown to the left of the figure are in kilodaltons.

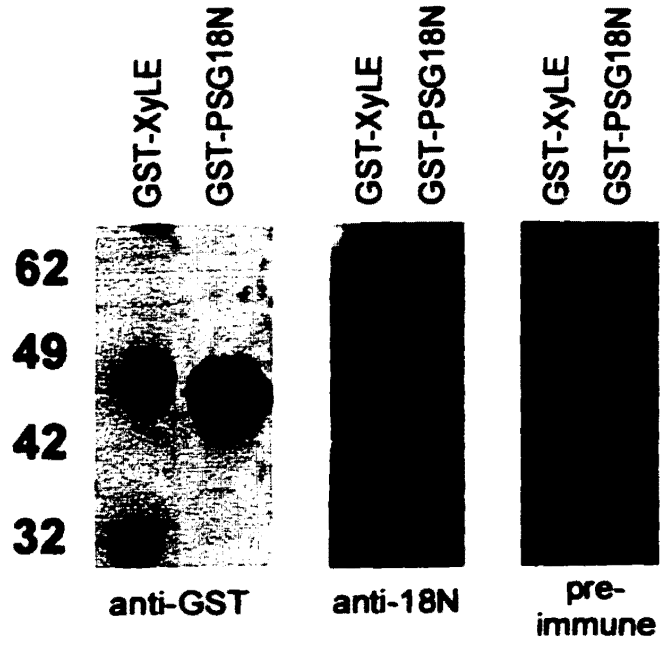
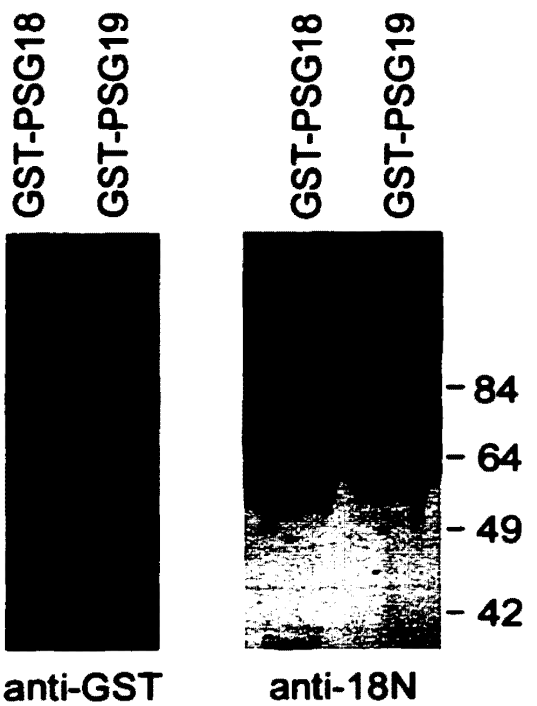

GST-XyLE
GST-PSG18N
GST-XyLE
GST-PSG18N
GST-XyLE
GST-PSG18N
62
49
42
32
anti-GST
anti-18N
pre-
immune

Figure 11. **The antibody to PSG18N also reacts with full length PSG18 and 19.** *Hi5* **supernants containing either GST-PSG18, or GST-PSG19 were electrophoresed in duplicate on 4-20% NuPAGE gels and transferred to nitrocellulose. The presence of PSG18 and PSG19 was determined by incubation with both anti-GST and anti-PSG18N (pAbPSG18N), followed by HRP-conjugated goat anti-mouse, or anti-rabbit, respectively. Both anti-GST and the anti-PSG18N reacts with the 80 kDa, full length fusion proteins, demonstrating the cross-reactivity of the polyclonal antibody. The molecular weight standards shown to the right of the figure are in kilodaltons.**


GST-PSG18
GST-PSG19
GST-PSG18
GST-PSG19
84
64
49
42
anti-GST
anti-18N

reactivity with the full length proteins. Figure 11 illustrates that both the anti-GST and pAbPSG 18N showed reactivity with the 83 kDa full length PSG18 and 19. In both Figure 10 and 11, it is evident that the polyclonal antibody show a high degree of non-specific binding. As a result, pAbPSG18N has only been used verify the presence of murine PSGs in baculovirus infected insect cell supernatants. Collaborators attempted to use the pAbPSG18N antibody for immunohistochemistry of uterine tissue, but as expected it resulted in a very high background (data not shown).

Functional studies using recombinant murine PSG18

It is widely accepted that cytokines have critical roles in ovulation, implantation, placentation, cervical dilation, and partruition (214). The anti-inflammatory environment that exists during pregnancy seems to be a protective mechanism, preventing an inflammatory or cell-mediated response that could potentially result in resorption of the fetal-placental unit (215). Placental trophoblast, uterine epithelium, and macrophages have been reported to synthesize IL-10 and IL-6. IL-6 is a cytokine that is likely to have a role in the tissue remodeling and in increasing the number of leukocytes in the uterus during pregnancy (214). Therefore, I hypothesized that PSGs act on macrophages to upregulate expression of cytokines associated with a successful pregnancy.

Thioglycollate-elicited murine peritoneal macrophages and RAW 264.7 cells, a murine macrophage cell line, were utilized to investigate the role of PSGs in regulating cytokine production. The full length murine PSGs were very difficult to produce and purify, therefore, purified GST-PSG18N or PSG18N-His were used for most of the experiments described in this section.

Both the full length and the N-terminal domain of PSG18 induce expression of IL-6 and IL-10 in RAW 264.7 cells and C3H/HeJ peritoneal macrophages.

The capacity of the full length GST-PSG18 and/or the truncated PSG18N to stimulate cytokine mRNA expression by RAW 264.7 cells (Figure 12,14) and thioglycollate-elicited C3H/HeJ peritoneal macrophages (Figure 13,15) was determined. Cell monolayers were treated with 10 μg/ml of recombinant PSG18, PSG18N, or the XylE control in both the presence and absence of 10 ng/ml protein-free *Escherichia coli* K235 LPS. Total cellular RNA was harvested at 2 hours post-treatment and expression of the house-keeping gene GAPDH, IL-6, and IL-10 mRNA were analyzed by RT-PCR. Since the full length proteins are so difficult to purify, the PSG18 used in this experiment was supernatant that was harvested from *Hi5* cells (in the presence of 5% fetal bovine serum) infected with PSG18 P4 virus and concentrated approximately 100-fold. The concentration of unpurified PSG18 present in the supernatant was determined as described in the methods section. In the absence of LPS, both PSG18 and PSG18N demonstrated a 5-10 fold increase of IL-10 mRNA and a 14-18 fold increase of IL-6 mRNA in

RAW 264.7 cells (Figure 12) and C3H/HeJ (Figure 13). For this and all subsequent experiments described, the GST-His-XylE and the untreated (data not shown) were always equivalent to 1. In addition, in the presence of LPS the XylE and untreated (data not shown) controls showed similar levels of IL-10 and IL-6 mRNA induction. The C3H/HeJ macrophages are extremely refractory to LPS stimulation, requiring greater than 1μg/ml LPS to activate minimal LPS-mediated signal transduction pathways (C. Salkowski, personal communication). C3H/HeJ macrophages were utilized for their altered ablility to respond to LPS. Together, the C3H/HeJ macrophage and RAW 264.7 cell data shown in Figure 12 and 13 suggest that the N1-domain is in fact sufficient to upregulate expression of IL-6 and IL-10 and that the observed induction in PSG-mediated.

Figure 12A shows that when RAW 264.7 cells were treated with PSG18 and PSG18N in the presence of 10 ng/ml LPS, there was a 93 fold and 58 fold upregulation of IL-10 expression over the XylE control, respectively. Similarly, a 120 and 95 fold increase of IL-6 mRNA over XylE control was observed for GST-PSG18 and GST-PSG18N, respectively. Treatment with LPS alone resulted in a 36 fold increase of IL-10 mRNA and a 90 fold increase of IL-6 mRNA. The data suggest that PSG18 and 18N may synergize with LPS to induce IL-10, but not IL-6 mRNA expression. No significant differences in IL-6 mRNA expression are observed when the RAW 264.7 cells are treated with 10 ng/ml of LPS in the presence or absence of PSG18N. It is possible that the 10 ng/ml of LPS saturates

Figure 12. PSG18 and PSG18N induced expression of IL-10 and IL-6 mRNA in RAW 264.7 cells. RAW 264.7 cells were treated with 10 μg/ml of XylE control, PSG18 and PSG18N alone, or in the presence of 10 ng/ml of LPS. mRNA was harvested at 2 hours post-treatment and IL-10 (A), IL-6 (B) and GAPDH mRNA were analyzed by RT-PCR. After reverse transcription, the cDNA were amplified with IL-10, IL-6, or GAPDH primer sets for 22, 25, and 14 cycles, respectively. Fifteen microliters of each PCR reaction was electrophoresed on 1.5% agarose and transferred to a nylon membrane using standard Southern blotting techniques. The hybridization signal for GAPDH, IL-10 and IL-6 was detected using the Storm PhosphoImager 860. IL-10 and IL-6 values were normalized against the GAPDH value. The fold increase was determined by comparing normalized values of the PSG treated sample to the XylE treated sample. For both (A) and (B), the solid bars designate treatments in the absence of LPS and the striped bars designate treatments in the presence of LPS. Data are expressed as the mean $\pm$ standard error of the mean (SEM) from three or four independent experiments. Asterisks indicate that the differences between the PSG and appropriate XylE control treatment were statistically significant ($P<0.05$).

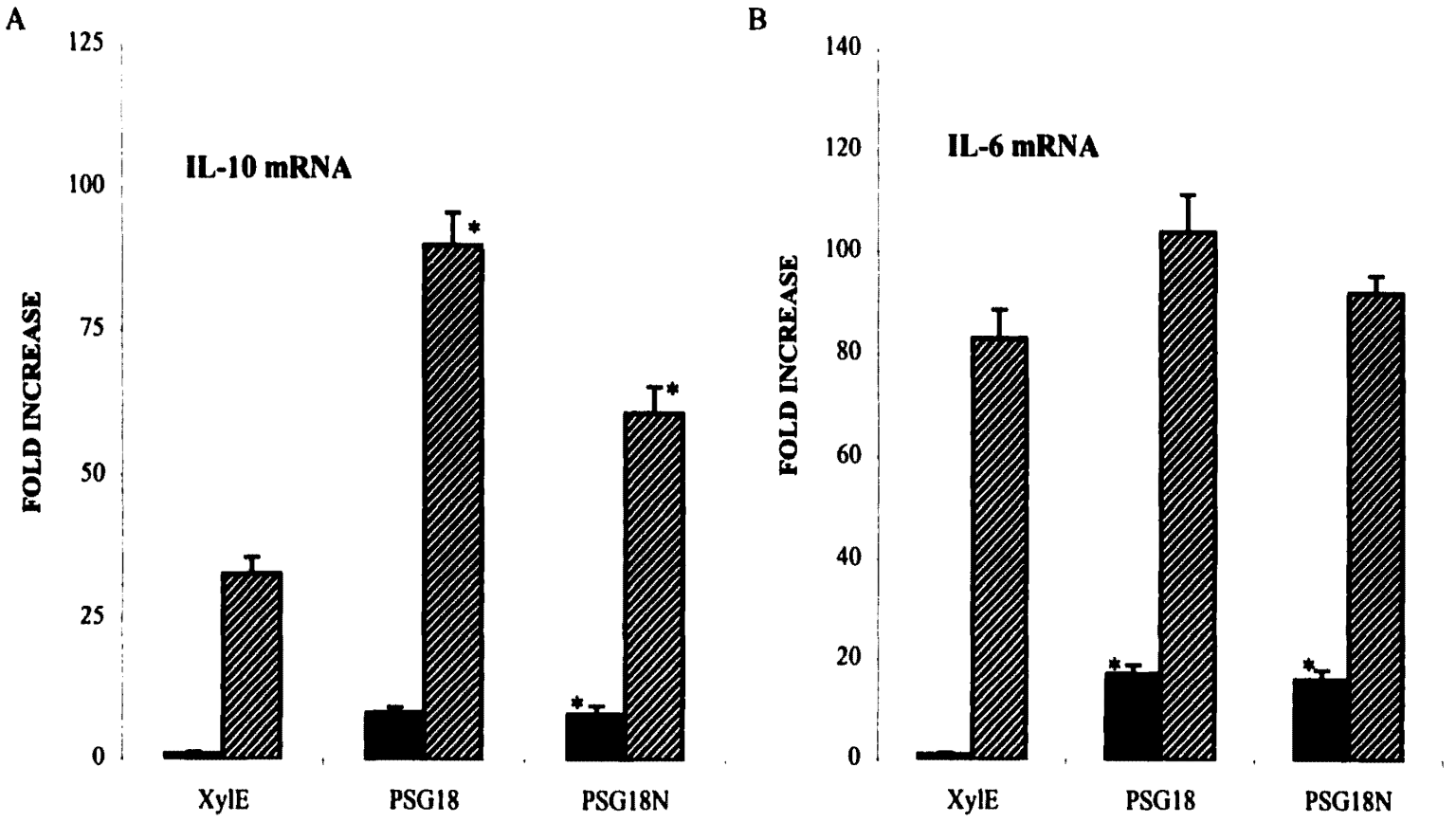
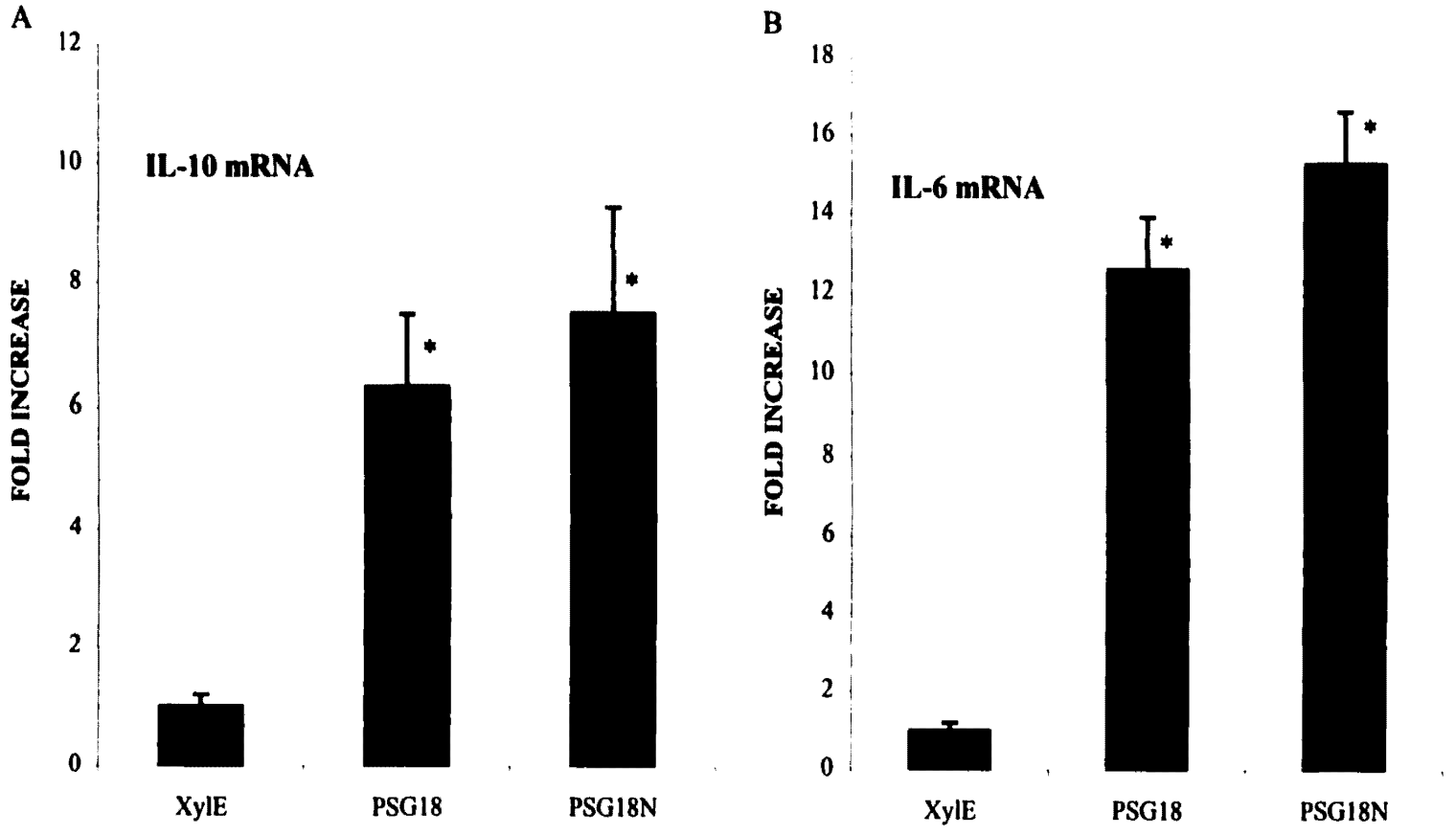

A
IL-10 mRNA
FOLD INCREASE
125
100
75
50
25
0
XylE
PSG18
PSG18N
B
IL-6 mRNA
FOLD INCREASE
140
120
100
80
60
40
20
0
XylE
PSG18
PSG18N

Figure 13. PSG18 and PSG18N induced expression of IL-10 and IL-6 mRNA in C3H/HeJ thioglycollate-elicited peritoneal macrophages. Cell monolayers were treated with 10 μg/ml of XylE control, PSG18, or PSG18N. mRNA was harvested at 2 hours post-treatment, reverse transcribed, and amplified with IL-10, IL-6, or GAPDH primer sets for 22, 25, and 14 cycles, respectively. Fifteen microliters of each PCR reaction was electrophoresed on 1.5% agarose gels, and transferred to a nylon membrane using standard Southern blotting techniques. The hybridization signal for GAPDH, IL-10 and IL-6 was detected using the Storm PhosphoImager 860. IL-10 and IL-6 values were normalized against the GAPDH value. The fold increase for IL-10 (A) and IL-6 (B) was determined by comparing normalized values of the PSG treated sample to the XylE treated sample. Data are expressed as the mean ± standard error of the mean (SEM) from three or four independent experiments. Asterisks indicate that the differences between the PSG and appropriate XylE control treatment were statistically significant ($P<0.05$).


A
IL-10 mRNA
FOLD INCREASE
12
10
8
6
4
2
0
XylE
PSG18
PSG18N
*
*
B
IL-6 mRNA
FOLD INCREASE
18
16
14
12
10
8
6
4
2
0
XylE
PSG18
PSG18N
*
*
*

IL-6 mRNA expression in RAW 264.7 cells, preventing further upregulation by PSG18N. These questions were investigated in greater detail at the protein level in RAW 264.7 cells, and will be addressed later.

The full length and N1-domain of PSG18 had similar patterns of expression of IL-6 and IL-10 mRNA, and as discussed previously, the full length proteins were extremely difficult to produce and purify. Therefore, only the PSG 18N was used for the remainder of the functional studies. Since, PSG18N-His and GST-PSG18N stimulated expression of IL-6 and IL-10 mRNA to a similar degree in both RAW 264.7 cells and peritoneal macrophages, the two fusion proteins were used interchangeably in all subsequent experiments.

To investigate whether PSG18N alters the expression of IL-1β, IL-12p40, TNF-α, TGF-β, and iNOS genes, RAW 264.7 cells (Figure 14) and C3H/HeJ macrophages (Figure 15) were treated for 2 hours with recombinant PSG18N or XylE in the presence or absence of 5 ng/ml LPS. In contrast to the IL-6 and IL-10 mRNA data described above, Figures 14 and 15 illustrate that PSG18N failed to induce expression of IL-1β, TNF-α, IL-12p40, iNOS, or TGF-β mRNA. In RAW 264.7 cells, LPS upregulated mRNA expression for all cytokines examined, except for TGF-β mRNA. However, treatment with LPS and PSG18N did not increase the expression of these immune mediators over that of LPS alone in RAW 264.7

Figure 14. PSG18N does not upregulate expression of IL-1β, IL-12, iNOS, TNF-α, or TGF-β mRNA in RAW 264.7 cells. Cell monolayers were treated with 10 μg/ml of PSG18N or XylE in the presence or absence of 5 ng/ml LPS and RNA was harvested 2 hours post-treatment. Expression of IL-1β, IL-12p40, iNOS, TNF-α, and TGF-β was analyzed by semi-quantitative RT-PCR for 17, 22, 20, 17, 30 cycles, respectively. Fifteen microliters of each PCR reaction was electrophoresed on 1.5% agarose gels, and transferred to nylon membrane using standard Southern blotting techniques. The hybridization signals were normalized against the GAPDH signal. The fold increase for IL-1β, IL-12p40, iNOS, TNF-α, and TGF-β was determined by comparing normalized values of the PSG treated sample to the XylE treated sample. Data are expressed as the mean + standard error of the mean (SEM) from three or four independent experiments. Asterisks indicate that the differences between the PSG and appropriate XylE control treatment were statistically significant ($P<0.05$). (N.D. = not detectable)

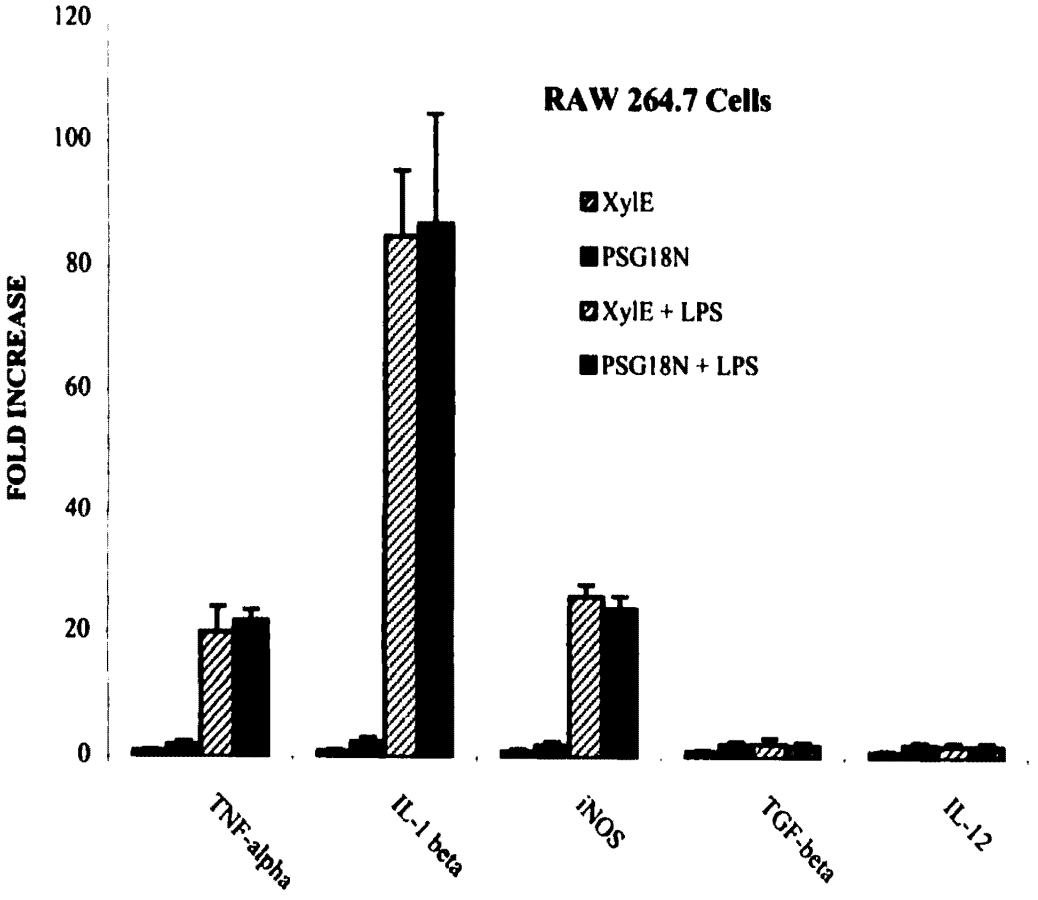
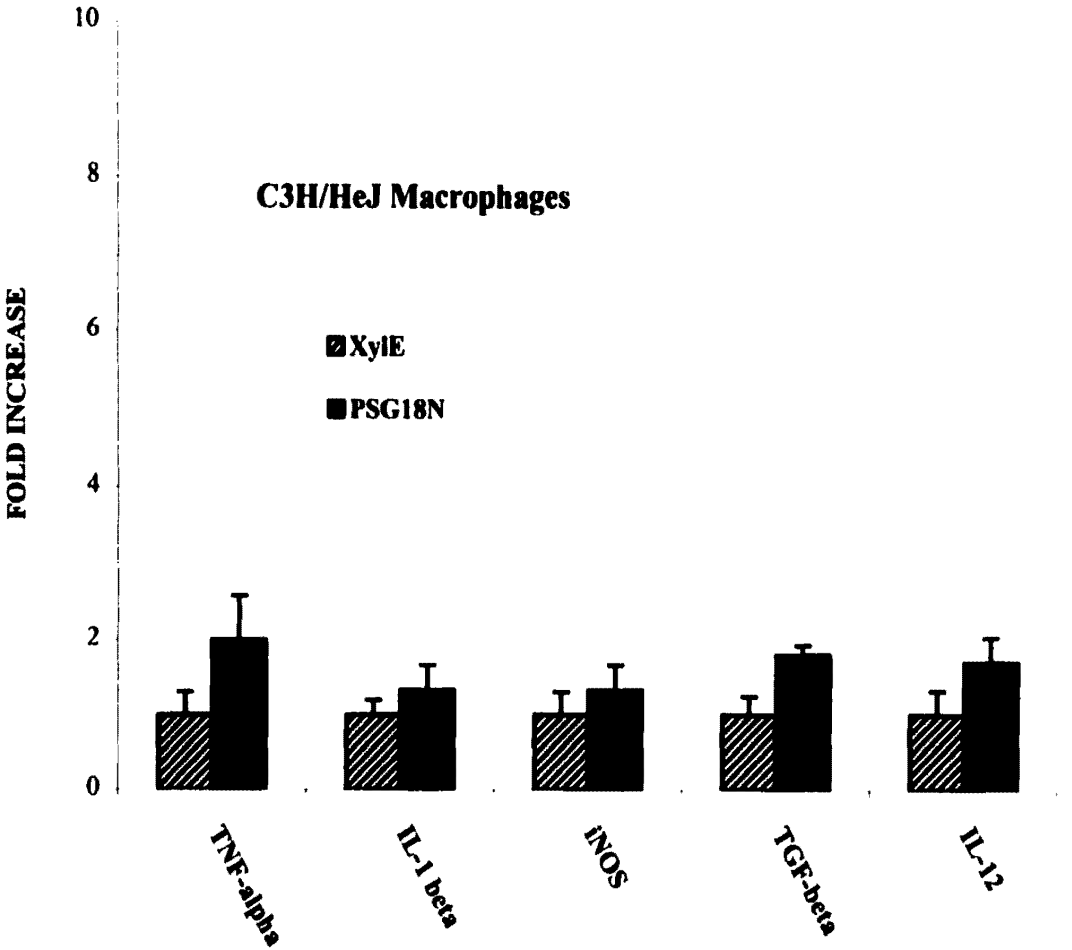

RAW 264.7 Cells
FOLD INCREASE
120
100
80
60
40
20
0
XylE
PSG18N
XylE + LPS
PSG18N + LPS
TNF-alpha
IL-1 beta
iNOS
TGF-beta
IL-12

Figure 15. PSG18N does not upregulate expression of IL-1β, IL-12, iNOS, TNF-α, or TGF-β mRNA in C3H/HeJ peritoneal macrophages. Cell monolayers were treated with 10 μg/ml of PSG18N or XylE for 2 hours. mRNA was harvested, reverse transcribed and amplified with IL-1β, IL-12p40, iNOS, TNF-α, and TGF-β primer sets for 17, 22, 20, 17, 30 cycles, respectively. Fifteen microliters of each PCR reaction was electrophoresed on 1.5% agarose gels, and transferred to nylon membrane using standard Southern blotting techniques. The hybridization signals were normalized against the GAPDH signal and the fold increase for IL-1β, IL-12p40, iNOS, TNF-α, and TGF-β determined by comparing normalized values of the PSG treated sample to the XylE treated sample. Data are expressed as the mean ± standard error of the mean (SEM) from three independent experiments. Asterisks indicate that the differences between the PSG and appropriate XylE control treatment were statistically significant ($P<0.05$).


C3H/HeJ Macrophages
FOLD INCREASE
10
8
6
4
2
0
XylE
PSG18N
TNF-alpha
IL-1 beta
iNOS
TGF-beta
IL-12

cells. Collectively, my results show that PSG18 and 18N only upregulate IL-6 and IL-10, cytokines that are known to be helpful to pregnancy (98).

Kinetics of IL-6 and IL-10 mRNA expression induced by PSG18N.

To examine the kinetics of IL-6 and IL-10 mRNA expression by PSG18N in RAW 264.7 (Figure 16) and C3H/HeJ thiogylcollate-elicited peritoneal macrophages (Figure 18), cell monolayers were incubated with 10 μg/ml of PSG18N or XylE in the presence or absence of 5 ng/ml of LPS. RNA was harvested at 1, 2, 4, 6 and 12 hours post-treatment and expression of GAPDH, IL-6, and IL-10 was analyzed by RT-PCR. Both the XylE and the untreated samples showed background levels of IL-6 and IL-10 mRNA expression. The absence of induction is given a value of 1. In both RAW 264.7 cells and C3H/HeJ macrophages, induction of IL-6 and IL-10 mRNA was detected as early as one hour post-treatment with PSG18N, and peaked at 2 hours. By 12 hours post PSG18N treatment, the level of IL-10 and IL-6 mRNA returned to baseline level. In RAW 264.7 cells (Figure 16), PSG18N treatment showed a similar trend of IL-10 expression regardless of the presence of 5 ng/ml LPS. However, the addition of LPS significantly increased the level of mRNA induction at all time points examined. Treatment with LPS alone demonstrated slightly different kinetics. Both IL-10 and IL-6 mRNA peaked at 4 hours in response to

Figure 16. Kinetics of IL-10 and IL-6 mRNA expression in RAW 264.7 cell. Cell monolayers were incubated with 10 μg/ml PSG18N or XylE control for the indicated times (1-12 hours). The treatments were performed in the presence and absence of 5 ng/ml LPS. mRNA was harvested at 2 hours post-treatment, reverse transcribed, and amplified with IL-10, IL-6, or GAPDH primer sets for 22, 25, and 14 cycles, respectively. Fifteen microliters of each PCR reaction was electrophoresed on 1.5 % agarose gels, and transferred to a nylon membrane using standard Southern blotting techniques. The hybridization signal for GAPDH, IL-10 and IL-6 was detected using the Storm PhosphoImager 860. IL-10 and IL-6 values were normalized against the GAPDH value. The fold increase for IL-10 (A) and IL-6 (B) was determined by comparing normalized values of the PSG treated sample to the XylE treated sample. Normalized XylE values are considered to be background levels IL-6 and IL-10 expression and are given a value of 1. LPS treatment peaked at 4 hours, while the mRNAs peaked at 2 hours in response to treatment with PSG18N. In addition, the induction observed for LPS alone was identical to that observed for XylE + LPS (A, B). Data are expressed as the mean $\pm$ standard error of the mean (SEM) from three independent experiments. Asterisks indicate that the differences between the PSG and appropriate XylE control treatment were statistically significant ($P<0.05$).

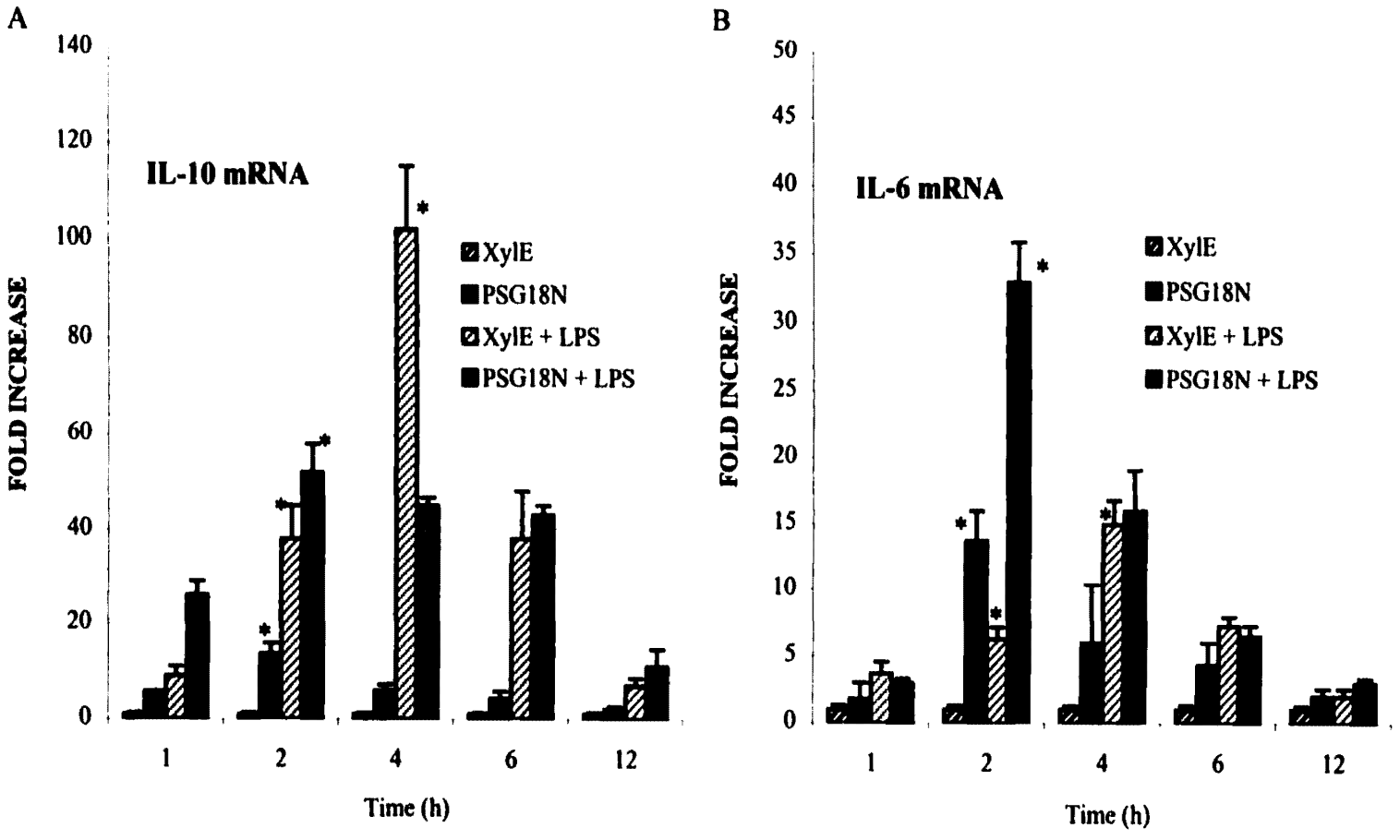

A
IL-10 mRNA
FOLD INCREASE
140
120
100
80
60
40
20
0
1
2
4
6
12
Time (h)
XylE
PSG18N
XylE + LPS
PSG18N + LPS
B
IL-6 mRNA
FOLD INCREASE
50
45
40
35
30
25
20
15
10
5
0
1
2
4
6
12
Time (h)
XylE
PSG18N
XylE + LPS
PSG18N + LPS

Figure 17. **Detection of increased IL-10 and IL-6 mRNA expression in RAW 264.7 cells. Southern blots of IL-10, IL-6 and GAPDH RT-PCR amplified products from the same representative experiment depicted in Figure 17. RAW 264.7 cell monolayer were treated with XylE (lanes 1 and 4) or PSG18N (lanes 2 and 3) in the presence (lanes 1,2) or absence (lanes 3, 4) of 5 ng/ml LPS. mRNA was harvested at the times indicated.**

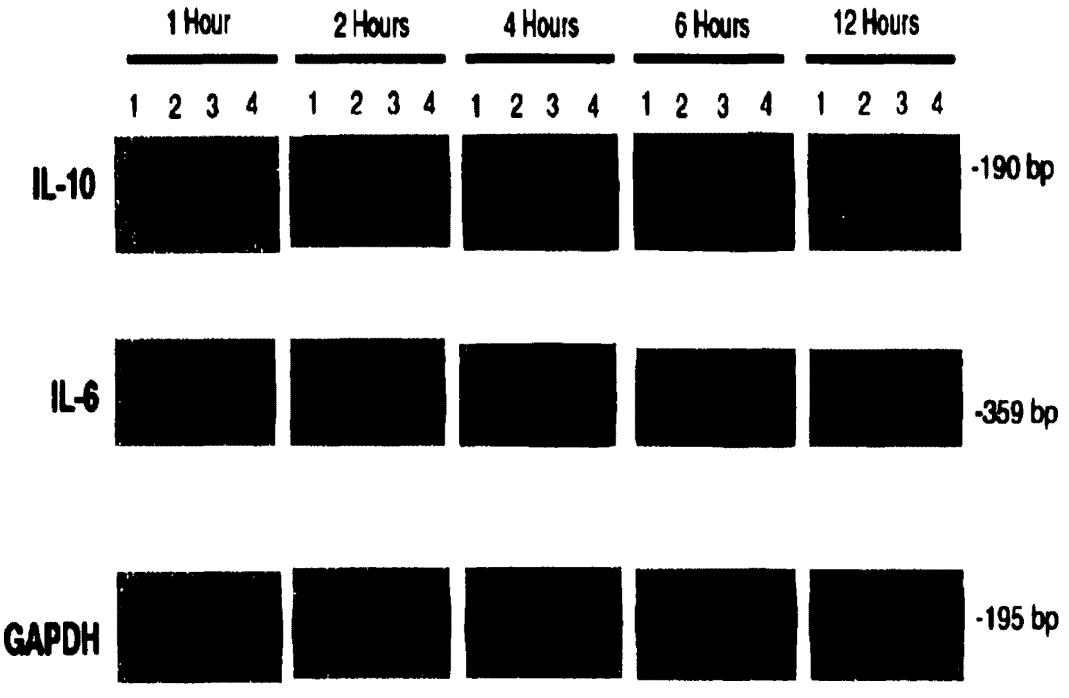
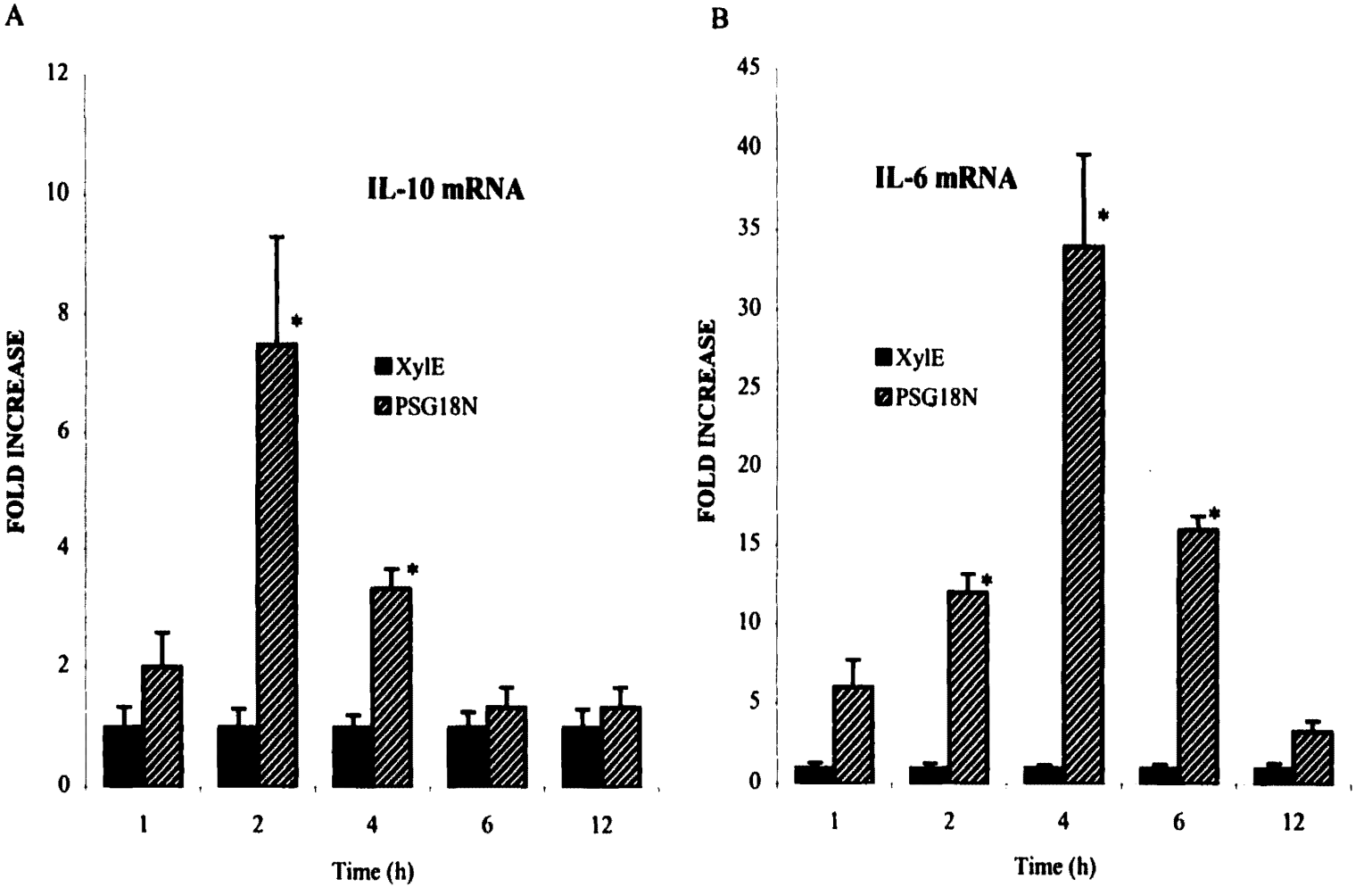

1 Hour
2 Hours
4 Hours
6 Hours
12 Hours
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
IL-10
-190 bp
IL-6
-359 bp
GAPDH
-195 bp

Figure 18. Kinetics of IL-10 and IL-6 expression in thioglycollate-elicited C3H/HeJ peritoneal macrophage. Cell monolayers were incubated with 10 ug/ml PSG18N or XylE control for the indicated times (1-12 hours). mRNA was harvested at 2 hours post-treatment, and IL-10, IL-6, or GAPDH expression was anaylzed by RT-PCR for 22, 25, and 14 cycles, respectively. Fifteen microliters of each PCR reaction was electrophoresed on 1.5 % agarose gels, and transferred to a nylon membrane using standard Southern blotting techniques. The hybridization signal for GAPDH, IL-10, and IL-6 was detected using the Storm PhosphoImager 860. IL-10 and IL-6 values were normalized against the GAPDH value. Normalized XylE values are considered to be background levels IL-6 and IL-10 expression and are given a value of 1. The fold increase for IL-10 (A) and IL-6 (B) was determined by comparing normalized values of the PSG treated sample to the XylE treated sample. Data are expressed as the mean $\pm$ standard error of the mean (SEM) from three independent experiments. Asterisks indicate that the differences between the PSG and appropriate XylE control treatment were statistically significant ($P<0.05$).


A
IL-10 mRNA
FOLD INCREASE
XylE
PSG18N
Time (h)
0
2
4
6
8
10
12
1
2
4
6
12
*
B
IL-6 mRNA
FOLD INCREASE
XylE
PSG18N
Time (h)
0
5
10
15
20
25
30
35
40
45
1
2
4
6
12
*

LPS treatment, while the IL-10 and IL-6 mRNAs peaked at 2 and 4 hours, respectively, in response to treatment with PSG18N. In addition, treatment of RAW 264.7 cell with PSG18N in the presence of LPS resulted in a shift of the kinetics of IL-6 and IL-10 mRNA expression as compared to treatment with LPS alone. Figure 17 shows a Southern blot analysis of the experiment depicted in Figure 16.

PSG18N induces secretion of IL-6 and IL-10 protein in a dose dependent manner.

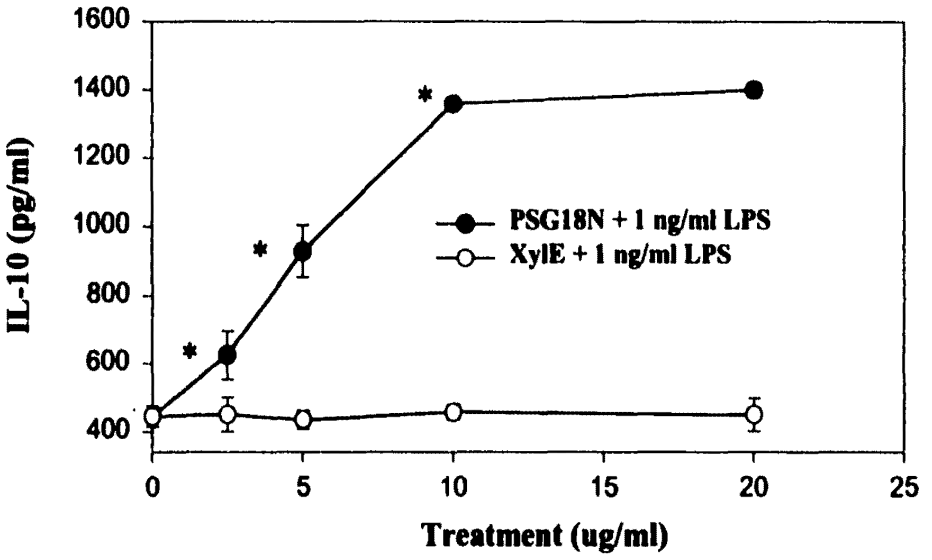
A dose response analysis was performed to determine the sensitivity of RAW 264.7 cells (Figure 19) and BALB/c thioglycollate-elicited peritoneal macrophages (Figure 20) to PSG18N. Increasing amounts of PSG18N or XylE, from 2.5 μg/ml to 20 μg/ml, were used to treat RAW 264.7 or BALB/c peritoneal exudate cell monolayers. In 24 hour supernatants, treatment with doses up to 10 ug/ml PSG18N in the absence of LPS did not cause an increase of IL-10 protein by either cell type. However, RAW 264.7 cells treated with 20 μg/ml of PSG18N or higher resulted in a significant induction of IL-6 and IL-10 protein (Figure 21). In BALB/c macrophages, doses as high as 40 μg/ml of PSG18N did not cause any change in levels IL-6 or IL-10 protein as compared to the XylE control. As shown in Figures 19A and 20A, treatment with 2.5 μg/ml to 20 μg/ml of PSG18N in combination with 1 ng/ml for RAW 264.7 cells or 100 ng/ml LPS for BALB/c

macrophages, showed a significant increase of IL-10 protein production. Induction of IL-6 protein by PSG18N was demonstrated to be dose dependent in RAW 264.7 cells (Figure 19B).

In BALB/c peritoneal macrophages, PSG18N treatment in the presence and absence of 100 ng/ml LPS resulted in a similar level of IL-6 protein. It seems likely that 100 ng/ml of LPS maximizes the IL-6 response, but not the IL-10 response in BALB/c macrophages. As shown in Figure 19, there was not a significant difference in the amount of IL-6 or IL-10 secreted by cells treated with 10 μg/ml as compared to 20 μg/ml of PSG18N in the presence of LPS. The level of IL-10 induction demonstrated in the cell line was dramatically greater than the induction observed in the peritoneal macrophages. This correlates with our observations that RAW 264.7 cells require at least 1 ng/ml of LPS to secrete significant amounts of IL-10, while peritoneal macrophages require LPS concentrations of 100 ng/ml. Together, the data suggest that peritoneal macrophages require a much greater stimuli to generate a detectable level of IL-10, than RAW 264.7 cells. In peritoneal macrophages, low levels of IL-10 protein despite high levels of IL-10 mRNA have been observed for many macrophage activators, including LPS (216). In addition, IL-10 protein has been shown to bind to the IL-10 receptor on the surface of the macrophages, and therefore, is difficult to detect in the supernatant of these cells (217).

Figure 19. PSG18N dose response in RAW 264.7 cells. Cell monolayers were treated with the indicated concentration (2.5–20 μg/ml) of PSG18N or XylE control in the presence of 1 ng/ml LPS. Cell culture supernatants were collected at 24 hours post-treatment and assayed for IL-10 or IL-6 protein by ELISA. PSG18N mediated induction of IL-10 (A) and IL-6 (B) is shown to be dose dependent. Treatment with LPS alone produced IL-10 protein values equivalent to the values shown for XylE in the presence of LPS. Data are expressed as the mean ± standard error of the mean (SEM) of treatments performed in triplicate and are representative of results obtained from three independent experiments. When not visible, SEM bars are smaller than the symbol. Asterisks indicate that the differences between increasing PSG treatments were statistically significant ($P<0.05$).

A


1600
1400
1200
1000
800
600
400
IL-10 (pg/ml)
0
5
10
15
20
25
Treatment (ug/ml)
PSG18N + 1 ng/ml LPS
XylE + 1 ng/ml LPS
*
*
*

B

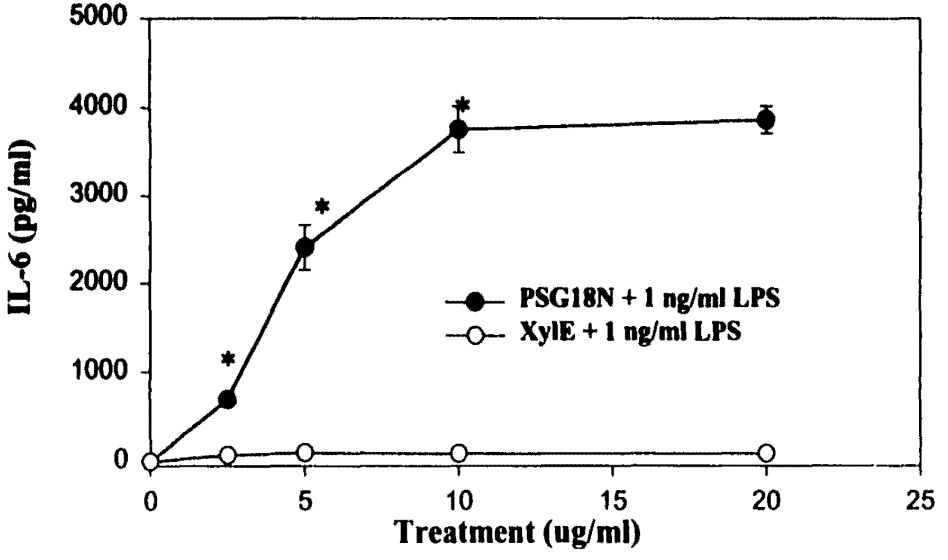
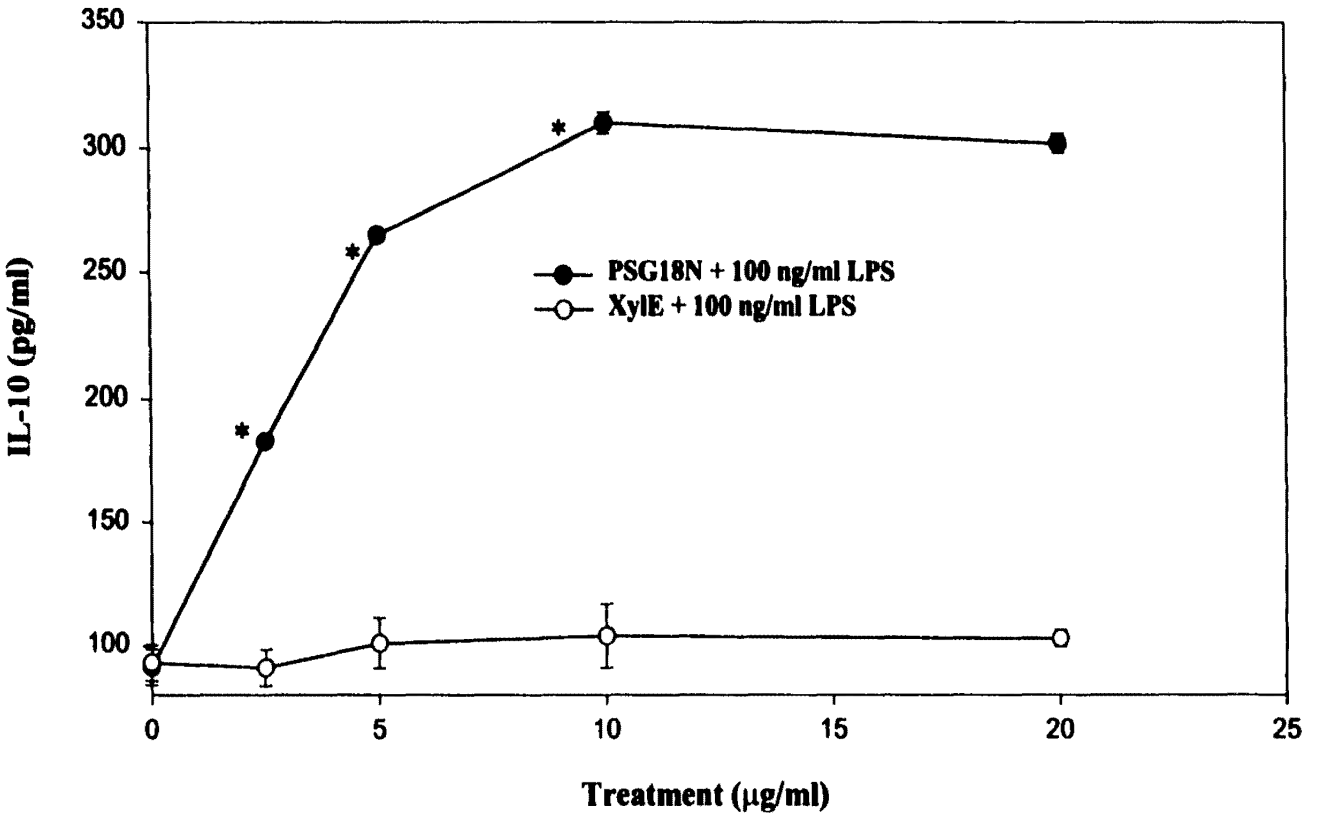

5000
4000
3000
2000
1000
0
IL-6 (pg/ml)
0
5
10
15
20
25
Treatment (ug/ml)
PSG18N + 1 ng/ml LPS
XylE + 1 ng/ml LPS
*
*
*

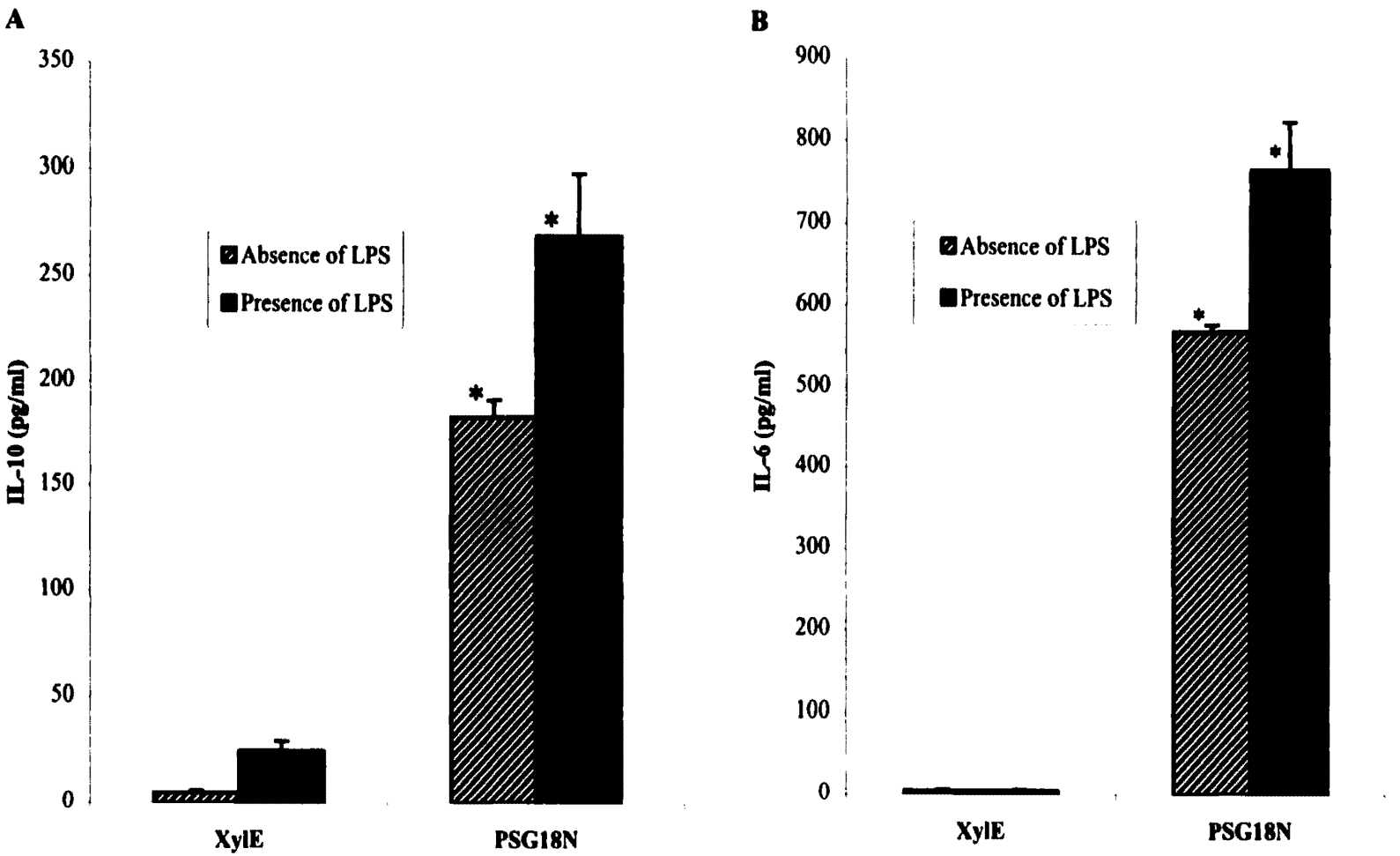
Figure 20. PSG18N dose response in BALB/c thioglycollate-elicited peritoneal macrophages. Cell monolayers were treated with the indicated concentration (2.5–20 μg/ml) of PSG18N or XylE control in the presence of 100 ng/ml LPS. Cell culture supernatants were collected at 24 hours post-treatment and assayed for IL-10 protein by ELISA. PSG18N mediated induction of IL-10 protein was shown to be dose-dependent. Treatment with LPS alone produced IL-10 protein values equivalent to the values shown for XylE in the presence of LPS. Data are expressed as the mean ± standard error of the mean (SEM) of treatments performed in triplicate and are representative of results obtained from three independent experiments. When not visible, SEM bars are smaller than the symbol. Asterisks indicate that the differences between increasing PSG treatments were statistically significant ($P<0.05$).


350
300
250
200
150
100
IL-10 (pg/ml)
PSG18N + 100 ng/ml LPS
XylE + 100 ng/ml LPS
*
*
*
0
5
10
15
20
25
Treatment (μg/ml)

PSG18N demonstrates synergy with LPS in RAW 264.7 Cells

Since RAW 264.7 cells seem to generate greater amounts of IL-6 and IL-10 in response to PSG18N treatment, they were used to address the question of synergy. First, the LPS amount used to treat the cells was varied to determine the dose of LPS required to be suboptimal for the production of IL-10 and IL-6 protein. For both cytokines, 0.5 ng/ml LPS was determined to be suboptimal. Therefore, RAW 264.7 cells were treated with 20 μg/ml XylE or PSG18N in the presence or absence of 0.5 ng/ml LPS, and assayed for IL-10 (Figure 21A) and IL-6 (Figure 21B) protein. In the absence of LPS, PSG18N treatment caused an increase in IL-6 and IL-10 levels over both the XylE and LPS control. In the presence of 0.5 ng/ml of LPS, treatment with 20 μg/ml PSG18N caused a significant increase in IL-6 and IL-10 protein over XylE, PSG18N or LPS alone. As shown in Figures 19 and 20, treatment with only 10 μg/ml PSG18N resulted in no upregulation of IL-6 or IL-10 in BALB/c or RAW 264.7 cells. Together, these results indicate that PSG18N may synergize with LPS to produce IL-6 and IL-10 protein in RAW 264.7 cells.

Figure 21. PSG18N synergizes with LPS to upregulate secretion of IL-10 and IL-6. RAW 264.7 cell monolayers were treated with 20 μg/ml of PSG18N or XylE control in the presence and absence of a suboptimal dose of LPS. Previous data indicated that 0.5 ng/ml of LPS was suboptimal for production of IL-10 and IL-6 protein in RAW 264.7 cells. Cell culture supernatants were collected at 24 hours post-treatment and assayed for IL-10 or IL-6 protein by ELISA. Both in the presence and absence of LPS, XylE treatment shows very little IL-10 (A) or IL-6 (B) protein (< 25 pg/ml). Treatment with PSG18 alone shows an induction of IL-10 and IL-6, but in the presence of LPS a greater induction of both cytokines is observed. Data are expressed as the mean ± standard error of the mean (SEM) of treatments performed in triplicate and are representative of results obtained from three independent experiments. When not visible, SEM bars are smaller than the symbol. Asterisks indicate that the differences between XylE control and PSG treatments were statistically significant ($P<0.05$).


A
350
300
250
200
150
100
50
0
IL-10 (pg/ml)
Absence of LPS
Presence of LPS
*
*
XylE
PSG18N
B
900
800
700
600
500
400
300
200
100
0
IL-6 (pg/ml)
Absence of LPS
Presence of LPS
*
*
XylE
PSG18N

IV. Discussion

The present study was undertaken to determine the ability of PSGs to alter maternal immunity by specifically upregulating the expression of anti-inflammatory cytokines during pregnancy. Although the concentration of murine PSGs in the serum of pregnant mice is unknown, the concentration of human PSGs reaches 200-400 μg/ml in the serum of pregnant women (5). This high concentration, along with reports that antibodies to PSGs induced abortions in mice and monkeys, suggests that PSGs may be critical to maintaining a successful pregnancy (14, 218). A murine cell culture model was established to examine whether recombinant PSGs regulate the expression of cytokines by macrophages, a cell type known to be prevalent in the uterus during pregnancy (142).

After cloning the full length *Psg18* and *19* cDNAs, their expression in tissues from adult mice was determined. Although the placenta was originally thought to be the only tissue to express *PSGs*, some extraplacental sites of *PSG* mRNA expression have been identified in humans. These sites include adult intestine, testis, and bone marrow-derived cells, and fetal liver (18, 20, 40, 219, 220). Wu and co-workers amplified RNA from PMNs, monocytes, T and B lymphocytes with *PSG*-specific primers for 28 to 46 cycles. A product was only detectable in RNA from T lymphocytes (221). However, previous immunohistochemistry experiments did not produce similar results (222). Zimmerman and co-workers screened libraries from human fetal tissues for the presence of *PSG*-like cDNA clones. Northern blot analysis with isolated cDNAs

was utilized to confirm that the *PSG* transcripts were present in the fetal liver (21). The complex splicing pattern that exists for most the *PSGs*, suggests that PSGs may have several different functions. Northern blot analysis was also utilized to demonstrate expression of *PSG* mRNAs in human testis (20). In addition, Western blot analysis showed that anti-human SP1 antibodies react with proteins present in normal testicular tissue. Although the same *PSG* mRNAs exist in the placenta and the testis, Western blot analysis revealed that proteins of different size are found in the two tissues (20). Similar data was reported for expression of *PSGs* in the intestine (18). Furthermore, it is possible that the antibody utilized may cross react with proteins of the CEA gene family. In summary, there appears to be very little definitive evidence of protein production at sites other than the placenta.

As shown in Figure 3, only the embryo and placenta appear to express murine *Psg17*, *18*, and *19*. The other adult mouse tissues, including testes, intestine, liver, kidney, spleen, lung, brain, thymus, and ovary, show no expression of the three murine *Psgs* examined. Due to the possible contamination of embryonic tissue with tissue from the placenta, and the results of *in situ* hybridrization studies in which *Psg18* was not detected in embryonic tissue (Finkenzeller, personal communication), murine *Psgs* are likely to be specifically expressed only by the placenta. Since a large family of closely related *Psg* genes exists in the mouse, it is possible that the *Psg17*, *18*, and *19*-specific oligonucleotides used for the differential hybridization of the Southern blots may

detect additional, not yet completely sequenced *Psg* mRNAs. However, as far as investigated, the murine *Psg* genes are coordinately expressed by the placenta (W. Zimmermann, unpublished results).

To establish a means of generating purified PSGs for use in functional studies, recombinant full length PSG17, 18, 19 and a protein containing the N1-domain only of PSG18 (named PSG18N) were expressed as fusion proteins using a baculovirus expression system. Although the baculovirus system was chosen for its ability to produce high levels of protein, the production of full length murine PSGs was extremely inefficient. As is evident in the time course of PSG18 expression (Figure 4), the 80 kDa band present in the early time points that corresponds to full length PSG18 is replaced by two smaller proteins with molecular weights of 45 kDa and 30 kDa at the later time points. Similar results were obtained for full length PSG17 and 19. It is interesting that together, the two smaller proteins observed in the immunoblot using a monoclonal anti-GST antibody have the same molecular weight as the full length PSG18. Although no experiments were performed to determine the identity of the smaller proteins, it is likely that the 45 kDa band represents a protein that contains the N-terminal domain with the GST tag and 30 kDa band corresponds to a protein that consists of only the GST tag. The murine PSGs possess 12 amino acid, leader-like sequences (L2' and L3') located just prior to the start of the N2 and N3-domains. These sequences are referred to as "leader-like" because of their hydrophobic nature. It is possible that these sequences may interrupt translation, causing full

length PSGs to be poorly produced. In support of this argument, a truncated GST-PSG18N has a molecular weight of 45 kDa and is produced much more efficiently than the full length proteins in the baculovirus system. Other eukaryotic expression systems were also utilized in an attempt to generate the full length proteins more efficiently. Production of PSG17 as a Fc fusion protein in 293T cells also resulted in no detectable protein by immunoblot (Zimmerman, personal communication).

The conditions required to produce full length PSG17, 18, and 19 were highly variable and not reproducible, probably due to the leader-like sequences mentioned above. For this reason, the baculovirus system was used to generate PSG18N, a truncated version of PSG18 that consists of only the N-terminal domain and a GST or a 6x His tag. As shown in Figure 6, the N1-domains of PSG17, 18, and 19 are similar. These murine PSGs share a 55% amino acid homology, suggesting that PSGs may have a high level of functional redundancy. In addition to the sequence similarity, previous reports indicate that the exposed N-terminal domain of proteins, belonging to the CEA family, is critical for their interaction with respective targets (210-213). Human and mouse PSGs are structurally different, but they do share a similar N1-domain which provides further evidence that this domain has a role in determining PSG function. There has been some speculation that the function of human PSGs may be associated with the presence the Arg-Gly-Asp (RGD) tripeptide sequence in the N-domain of human PSG2, 3, 5, 6, 11, and 13 (51). This sequence is present in a variety of

extracellular matrix proteins and is critical for binding to integrin receptors (223). Murine PSG17 and 18 have an RGE sequence in the N1-domain that shares a common spatial and charge pattern with the RGD motif, while PSG19 does not possess a RGD-like motif within its N1-domain. If this motif has a role in receptor binding, then PSG17 and 18 would be expected to bind to a different receptor, and therefore have a different function from that of PSG19. However, at present it is not clear whether these motifs have any role in the functional activity of human or murine PSGs.

Recombinant GST-PSG17, 18, and 19 (Figure 5) have molecular weights of 80-85 kDa, while the GST-PSG18N and PSG18N-His (Figure 7) have a molecular weight of 45 kDa and 23 kDa, respectively. Murine PSGs possess 6-7 potential N-linked glycosylation sites, 3-4 of which are located in the N-terminal domain. The size of the PSG17, 18, 19 and 18N fusion proteins, as determined in the anti-GST or anti-His immunoblot, suggests that they are glycosylated when produced in insect cells. As shown in Figures 5 and 7, precipitation with Concanavalin-A-Sepharose demonstrated that both the full length and truncated N1-domain proteins possess some degree of glycosylation. Concanavalin A will bind any molecule that possesses a carbohydrate moiety in the correct configuration. In Figure 5, there are doublets present in the lanes corresponding to Concanavalin-A-Sepharose precipitated PSG18 and 19. This often occurs as a result of differential post-translational modifications, in this case probably due to different degrees of glycosylation.

After establishing an efficient means of generating protein, a purification scheme was developed to generate PSG18N in an active conformation. Since no antibody to the murine PSGs was available, the PSGs were created with a GST/6x His tag for purification. Unfortunately, the murine PSGs generated in the baculovirus system and purified by affinity chromatography utilizing the encoded tag sequences were not biologically active. As indicated by preliminary human and mouse data, biological activity was determined by the ability of PSG18 to induce IL-10 mRNA expression by murine macrophages. The absence of biological activity could result from the protein being produced in an inappropriate conformation or being bound to other proteins, thus inhibiting receptor binding. After attempting many different ways to purify PSG18N, the scheme depicted in Figure 8 was developed. To obtain a homogeneous protein preparation with biological activity, *Hi5* supernatant containing PSG18N was denatured by SDS-polyacrylamide gel electrophoresis and SDS was removed, allowing some degree of renaturation. As shown in Figure 9, this method of purification resulted in a single band on a Coomassie-stained polyacrylamide gel. PSG18N, purified in this manner, was utilized to examine the effect of PSGs on murine macrophages.

As mentioned previously, no antibody to murine PSGs was available at the onset of this project. Recombinant GST-PSG18N was used to generate a rabbit polyclonal antibody. The antibody, pAbPSG18N, reacts with PSG18N (Figure 10), as well as full length PSG18 and 19 (Figure 11). The cross-reactivity with PSG 19 is not surprising due to the similarity of the N1-domains of all three

murine PSGs used in this study. The potential uses of such an antibody include: (1) neutralization of PSG18N function to show specificity in murine macrophages; (2) immunohistochemistry to determine which cells present in the placenta are responsible for secretion of PSGs; (3) determination of PSG size in serum obtained from pregnant mice; and (4) development of an ELISA to determine PSG serum concentration at different stages of pregnancy. Unfortunately, even after purification with a protein G column and a GST column, the antibody still demonstrated a high degree of non-specific binding. This could be attributed to the presence of antibodies that react with serum proteins. In the case of immunohistochemistry, incubation with pAbPSG18N produced very high background, making the result uninterepteble. As a result, the antibody was only used to verify the presence of murine PSGs in virus infected *Hi5* and *Sf9* cell supernatants.

The availability of an antibody to murine PSGs would make research in the field much easier. Others in the laboratory are in the process of generating enough PSG18N-His, a protein that is produced very efficiently in our system, to attempt immunizing male mice. Because PSGs are not produced in male mice, the animals should generate antibodies to the foreign antigen. An antibody that reacts with all PSGs would be extremely useful. In addition to the *in vivo* uses described above, there are several other potential uses of an anti-murine PSG. It could be used to: (1) examine the effect of PSG depletion on pregnancy outcome; (2) verify that PSGs are only present in the placenta; (3) examine the level of PSGs in

females of the abortion-prone mating model; and (4) examine the level of PSGs in IL-10 knock-out mice at different stages of gestation. This last experiment will address the question of whether PSGs possess a feedback mechanism. Since the IL-10 knock-out mice are not capable of producing IL-10, the placenta may continue to generate PSGs. In *in vitro* studies, the antibody could be used to detect and purify recombinant PSGs, to immunodeplete supernantants that contain PSGs, and to aid in isolation of the receptor. The antibody could be conjugated to a radioactive or flourescent label and used to detect PSG binding.

Results obtained from previous human studies are suggestive of a functional role for PSGs during pregnancy. A peptide derived from the N1-domain of PSG11 was shown to bind to cells of the promonocyte lineage (51). In addition, treatment of elutriated monocytes and the human monocyte cell line THP-1with PSG11 in the presence of LPS resulted in upregulated IL-10 expression (52). In the pregnant uterus, macrophages are found primarily in the connective tissue and meschymal stroma and the mesenchymal region of the placental villi (67, 131, 142). In humans, PSGs are synthesized shortly after implantation at the maternal-fetal interface (224-226). Implantation occurs at day 4.5 of gestation in mice. A recent study demonstates that PSG18 expression is detectable by *in situ* hybridization as early as day 6.5 of gestation, but earlier time points were not investigated (227). The spatiotemporal expression pattern of PSG18 correlates with the location of macrophages during pregnancy. Together, the data suggest that macrophages are a likely target for PSGs.

Unpurified PSG18 and purified PSG18N were used to investigate whether murine PSGs affected the pattern of cytokine production by murine macrophages. Since extraction of uterine macrophages is very difficult and only small quantities of these cells can be isolated per pregnant female, thioglycollate-elicited peritoneal macrophages from female mice and RAW 264.7 cells were utilized in this study. Macrophages are capable of producing a wide array of cytokines, growth factors and inflammatory mediators, including IL-1, IL-6, IL-10, IL-12, TNF-α, TGF-β, IFN-γ, macrophage colony stimulating factor (CSF-1), granulocyte-macrophage colony stimulating factor (GM-CSF), nitric oxide synthetase (iNOS), and prostaglandins (228). Murine thioglycollate-elicited peritoneal macrophages and the macrophage cell line 264.7 were treated with unpurifed PSG18 or purified PSG18N and examined for induction of various cytokines by RT-PCR and ELISA. Unpurified PSG18 was used because the full length proteins are nearly impossible to purify in adequate amounts for use in experiments. *Hi5* cells that were grown in 5% fetal bovine serum were used to generate full length PSG18. Collectively, my data suggest that serum may be necessary to produce proteins in an active conformation. For all experiments, a non-specific bacterial protein that possesses both a GST and 6x His tag, GST-His-XylE, was used as a negative control. The XylE control was purified in a similar manner to the PSGs. In addition to the XylE control, an untreated and a *Hi5* media with 5 % FBS in the presence and absence of LPS were also used as a negative controls (data not shown). In all experiments, the XylE, untreated and Hi5 media controls had no effect on cytokine production

and were set to equal 1. In the presence of LPS, the untreated (data not shown) and XylE controls showed similar levels of mRNA expression.

I determined that both the full length recombinant PSG18 and the N1-domain, PSG18N, upregulate IL-6 and IL-10 mRNA expression by RAW 264.7 (Figure 12) cell and C3H/HeJ thioglycollate-elicited peritoneal macrophages (Figure 13). In these cells, IL-10 and IL-6 mRNA expression are detectable at the 2 hour post-treatment with PSG18/18N. RAW 264.7 cells were also treated with PSG18N in the presence of polymyxinB, a polycationic antibiotic known to neutralize the effects of LPS *in vitro* (229). The results of these treatments were very similar to that of the C3H/HeJ macrophages. There was a 5-8 fold increase in IL-6 and IL-10 mRNA expression following treatment with PSG18N in the presence of polymyxinB (data not shown).

Murine macrophages are highly sensitive to LPS or endotoxin from Gram negative bacteria, resulting in the activation of a multitude of response pathways (228, 230). Macrophages from C3H/HeJ mice were utilized due to their altered ability to respond to LPS. The C3H/HeJ macrophages are hyporesponsive to LPS (231), requiring more than 1 μg/ml LPS to generate an increase in expression of cytokine mRNA (C. Salkowski, personal communication). The C3H/HeJ mice possess a missense mutation within the *Lps*^{d} or Toll-like receptor-4 gene (*Tlr4*) which causes a disruption of the LPS signal transduction pathway (232). LPS complexed with LPS binding protein (LBP) binds the monocyte/macrophage specific receptor, CD14 (233). Following direct interaction with LPS, the CD14

molecule engages *Tlr4*, allowing signal transduction to occur through this transmembrane molecule (232). In addition to using macrophages from C3H/HeJ mice, all PSG preparations were analyzed for LPS contamination with the *Limulus* amoeboctye lysate assay. Collectively, the data in C3H/HeJ peritoneal macrophages and RAW 264.7 cells indicates that the N-domain is critical to PSG function and that induction of IL-6 and IL-10 mRNA expression is a PSG-mediated event.

Further support for the functional role of PSGs is provided by the experiments shown in Figure 16, 17 and 18 which examined the kinetics of IL-6 and IL-10 expression following treatment with PSG18N. Treatment with LPS and PSG18N results in mRNA expression for these two cytokines that peaks at 4 hours and 2 hours, respectively (Figure 16, 17). The data suggest that different pathways are utilized for PSG-mediated and LPS-mediated induction of IL-6 and IL-10 mRNA expression. In addition the time course data indicates that the 2 hour time point in maximal for IL-10 and IL-6 mRNA expression in the RAW 264.7 cells and C3H/HeJ macrophages. This is important because it indicates that PSG-mediated induction is an early event, and therefore, may not require synthesis of an intermediate protein.

Proinflammatory cytokines have been shown to be harmful to pregnancy (98, 187, 189). Therefore, it is important to emphasize my results, which demonstrate that PSG18N treatment caused no induction of IL-1β, TNF-α, IL-12p40, or iNOS gene expression by RAW 264.7 cells (Figure 14) or murine peritoneal

macrophages (Figure 15) at the 2 hour time point. Also, PSG18N did not induce expression of TGF-β, a cytokine that has been shown to be beneficial to a successful pregnancy (101). LPS at a concentration of 5 ng/ml was used as a control in these experiments. In response to treatment with LPS, increased mRNA expression was readily detected for all of the previously mentioned cytokines at the 2 hour timepoint. However, IL-12p40 message was not detectable in RAW 264.7 cells, following treatment with the LPS control because these cells do not express IL-12p40 (personal communication, C.Salkowski).

In addition to the 2 hour time point, PSG-mediated induction of IL-1β, TNF-α, IL-12p40, and iNOS gene expression was also analyzed at 1 and 4 hours post-treatment (data not shown). The results were very similar to that shown in Figure 14 and 15. Since IL-10 protein was detectable in the supernatant of PSG18N treated macrophages and RAW 264.7 cells by 6 hours, later time points were not examined (data not shown). Further support of this decision is provided by several reports which indicate that IL-10 downregulates expression of these immune mediators by macrophages/ monocytes (120, 124, 234).

PSG18N also upregulates IL-6 and IL-10 protein in a dose dependent manner. Murine macrophages (Figure 19) require a substantial amount of LPS (100 ng/ml) in order to secrete detectable levels of IL-10 protein by ELISA. IL-10 protein is very difficult to detect in macrophage supernatants. Treatment of C3H/HeJ and C3H/OuJ macrophages with PSG18N resulted in IL-10 levels that

were barely within the range of detection of the assay (data not shown). For this reason, BALB/c mice were utilized because they are capable of producing more IL-10 than many other strains of mice (W. Gause, personal communication). One possible explanation for the difficulty in detection of IL-10 in macrophage supernatants is that IL-10 binds to the surface of macrophages in culture, dramatically decreasing the amount of soluble IL-10 (217).

Little to no difference in the level of IL-6 protein is observed following treatment of BALB/c macrophages with LPS in the presence or absence of PSG18N. My results indicate that upregulation of IL-6 protein requires less LPS than upregulation of IL-10. Therefore, it seems likely that 100 ng/ml of LPS saturates the macrophage IL-6 signal transduction pathway, preventing further upregulation by a secondary stimulus such as PSG18N. In contrast to macrophages, treatment of RAW 264.7 cells (Figure 20) with PSG18N in the presence of 1 ng/ml LPS stimulates secretion of IL-6 and IL-10. In the presence of LPS, treatment with 10-20 μg/ml of PSG18N appears to maximize secretion of IL-6 and IL-10. It is interesting to note that in the absence of LPS, treatment of RAW 264.7 cells with less than 20 μg/ml of PSG18 results in little or no induction of either IL-6 or IL-10 (data not shown). Treatment of RAW 264.7 cells with a suboptimal dose of LPS (0.5 ng/ml) and 20 μg/ml of PSG18N demonstrates a level of IL-6 and IL-10 protein greater than treatment with either PSG18N or LPS

alone (Figure 21). Therefore, LPS may synergize with PSG18N, potentiating the induction of IL-6 and IL-10 by RAW 264.7 cells.

Collectively, these data suggest that *in vitro*, peritoneal macrophages and RAW 264.7 cells require a primary stimulus in order to respond to PSGs. At present, it is not known whether macrophages at the maternal-fetal interface respond to PSGs in a manner similar to these *in vitro* studies. It is possible that macrophages in the pregnant uterus are already primed, more readily secreting IL-6 and IL-10 in response to PSGs than circulating monocytes or peritoneal macrophages. During pregnancy, blood monocytes migrate to certain anatomic compartments within the uterus (134). Once in the tissue, the local uterine environment stimulates expression of activation associated markers causing the differentiation of monocytes into phenotypically distinct subpopulations (96, 235). It is likely that factors prevalent at the maternal fetal interface may initiate monocyte maturation, generating a unique population of activated macrophages. For this reason, the LPS utilized in my experiments is probably replaced by immune mediators such as TGF-β, IL-4, or progesterone *in vivo*.

Progesterone, TGF-β, and IL-4 are all present at greater concentrations in the pregnant as compared to the cycling uterus (138, 236). Bioactive TGF-β, a suppressor cytokine, is present in the murine decidua and has been shown to enhance the ability of macrophages to produce IL-10 (237-239). In addition, progesterone-induced blocking factor (PIBF), a protein that mediates the

immunologic effects of progesterone, is associated with elevated levels of IL-10 at the maternal-fetal interface (240, 241). Once "primed" with one of these factors, uterine macrophages are then sensitive to being triggered by secondary signals present within the local feto-placental environment (146). I suggest that PSGs may function as a secondary signal for these "primed" macrophages. Therefore, the question of whether treatment of murine macrophages and RAW 264.7 cells with PSG18 in the presence of TGF-β, IL-4, or progesterone stimulates secretion of IL-6 and IL-10 protein in a manner similar to that of LPS must be addressed.

IL-6 is 26 kDa, multifunctional cytokine that is produced by a variety of cell types including endothelial cells, trophoblast cells and uterine macrophages (242). Its biological roles include regulation of hematopoiesis, proliferation and function of B and T lymphocytes and macrophages, and participation in acute-phase reaction (243). Although it shares a variety of biological activities with leukemia inhibitory factor (LIF), TNF-α, and IL-1β (244, 245), IL-6 seems to have a variety of potential roles during pregnancy. Many cells including macrophages have been shown to differentiate or proliferate in response to IL-6. Studies indicate that IL-6 stimulates cultured placental cells to produce human chorionic gonadotropin (hCG) and human placental lactogen (hPL) (246, 247). In addition, IL-6 is likely to have a role in the tissue remodeling and the influx of leukocytes in the uterus (214). Studies in mice suggest that IL-6 bioactivity is maximal at days 5 and 6 of gestation, indicating that IL-6 may have a role in the implantation process (248, 249). Further support for the role of IL-6 in tissue

remodeling during implantation is provided by the fact that macrophages and trophoblast cells possess IL-6 receptors which suggests that they may be a potential target for IL-6 action (250, 251). More recently, IL-6 was shown to inhibit the production of IL-1β and TNF-α by murine decidual cell cultures (245, 252) and uterine macrophages (253). This evidence suggests that, much like IL-10, IL-6 may have a role in suppressing the expression of cytokines that are potentially harmful to the developing fetus.

IL-10 is present at high levels during pregnancy and it has been postulated that the local secretion of IL-10 contributes to the generation of a maternal immune state compatible with a successful pregnancy (99, 194). IL-10 inhibits the expression of class II MHC and B7 molecules on macrophages and makes them unable to function as accessory cells for natural killer (NK) cells resulting in diminished IFN-γ production (124, 254-256). Interestingly, induction of NK and lymphokine-activated killer (LAK) cell activity has been linked to spontaneous fetal resorption in mice (257). Besides being a cytokine with a wide range of anti-inflammatory properties, IL-10 has been shown to inhibit human placental cytotrophoblast invasiveness and metalloproteinase production in human mononuclear phagocytes (71, 258). Sources of IL-10 during pregnancy include the human placenta and supernatants derived from the murine feto-placental unit (129, 130, 259). Macrophages both produce and respond to IL-10 (254).

During pregnancy, uterine NK or GMG cells are almost as prevalent as macrophages at the maternal-fetal interface (260). It is important that future

research be directed at understanding the role of uterine NK cells and the possible function of PSGs with regard to these cells. Reports suggest that uterine NK cells belong the NK cell lineage, expressing typical markers such as LGL-1, asialo-GM1, and NK1.1 and containing cytolytic mediator such as perforin and serine esterases (155, 261). At present, it is not clear why cells that have the potential to be so harmful to the fetus are so prevalent. However, more recent experiments indicate that surface morphological characteristics visible by electron microscopy could be used to distinguish uterine NK cells from classical NK cells found in the speen (262). In humans, NK-like cells found in the uterus express VLA-1 (very late antigen 1) on their surface, while classical NK cells in the periphery do not express VLA-1 (263). In addition, less than 1% of circulating NK cells show the granulated decidual phenotype ($CD16^{-}CD56^{bright}$) (264). Together these results suggest that uterine NK cells are a transient population of functionally distinct cells.

Uterine NK cells in human and rodents are prevalent at the time of implantation, preferentially located around arteries (265). For this reason, it is likely the NK cells are crucial for the development of normal placentation. There specific localization is indicative of a role for uterine NK cells in the proper invasion of the decidua and the formation of the vasculature of the placenta which are necessary to provide the fetus with an uncompromised supply of oxygen and nutrients. Evidence of this is provided by the CD3ε transgenic mice which lack both NK and T cells (266). These mice showed an increased rate of fetal loss,

reduced placental size and low birth weights. In addition there has been much speculation that NK cells present at the implantation site may exert their effects through the production of cytokines (267). Cytokines produced by NK cells may influence the differentiation of trophoblast cells and macrophages, or aid in the regulation of the Th2 cell bias that exists during pregnancy. The precise function of this distinct population of NK cells remains unclear. However, the potential role of PSGs in influencing NK cells must be addressed. Since PSGs are prevalent by the time of implantation (227), it is possible that they may function to control NK cell mediated invasion of the trophoblast. They may accomplish this indirectly through their affect on macrophages or by direct interaction with the NK cells present at the implantation site.

Based on my results, I suggest that PSGs secreted by the placenta interact with macrophages, causing IL-6 and IL-10 to be produced during pregnancy. Reports indicate that IL-10, and perhaps IL-6, function to downregulate the proinflammatory cytokines, IL-1β, IFN-γ, and TNF-α (259, 268, 269). Therefore, PSGs may contribute indirectly to the downregulation of Th1-type or cell-mediated responses during pregnancy and may have a role in controlling placental invasion. In addition, it is interesting to speculate that PSGs may also bind to cytotrophoblasts and induce these cells to produce IL-6 and IL-10. Induction of IL-6 or IL-10 following treatment of trophoblast cells with PSGs needs to be investigated. Also, it will be interesting to determine whether the same mechanism is utilized by PSG-treated macrophages to upregulate expression of

IL-6 and IL-10. I suspect that these two cytokines perform different functions during pregnancy, and that different pathways could regulate their production.

In the past 50 years there have been theories that address the immunologic phenomenon of pregnancy, and although my research suggests that PSGs are a critical factor, there are still many unanswered questions. PSG18N specifically upregulates IL-6 and IL-10 mRNA and protein expression in peritoneal macrophages and the murine macrophage cell line RAW 264.7. PSG-mediated induction of cytokines is an early event and is dose dependent. Together these data strongly suggest that the biological activity of PSG18 is receptor-mediated, and that the receptor must be expressed on the surface of macrophages. Further evidence for the presence of a receptor in both macrophages and RAW 264.7 cells is provided by binding studies performed by others in my laboratory. One of the primary goals of this laboratory will be to clone the PSG receptor present on the surface of murine macrophages. As mentioned earlier, the existence of an anti-PSG antibody would make this easier. In the absence of an antibody, a murine macrophage expression library will be screened with recombinant PSG18N-His in order to isolate the PSG receptor. Identification of the PSG receptor will help in the elucidation of the signaling pathways responsible for PSG-mediated IL-6 and IL-10 induction.

I have shown that PSGs do have a role in modulating the maternal immune system, but it is possible that PSGs may have more than one function. PSG may also have roles in cellular adhesion, or in stimulation and inhibition of growth.

Others in the laboratory have generated truncated versions of PSG17 and PSG19 consisting only of the N-terminal domain and a 6x Histidine tag. It would be interesting to see whether all three proteins have a role in modulating cytokine production by murine macrophages. Since PSG19 does not possess an RGD-like motif within its N1-domain, this experiment would also address the question of whether the motif plays a role in receptor binding. Also, it is important to know whether PSGs modulate cytokine expression, or promote growth of cells present at the maternal-fetal interface during pregnancy. For this reason, uterine macrophages, cytotrophoblast cell, uterine NK cells, and uterine epithelial cells should be treated *in vitro* with PSGs and cytokine production determined. The cells should also be examined for proliferation in response to PSG treatment.

Regulation of T-cell dependent immune responses and macrophage reprogramming are two routes by which PSGs may aid the fetus in escaping immune rejection. Signaling that occurs through the CD40-CD40 ligand (CD40L) and B7-CD28 pathways are essential to the maintnance of normal host immunity (270, 271). In the first pathway, CD40 present on the cell surface of antigen presenting cells interacts with CD40L (CD154) expressed on activated T cells. In the B7-CD28 pathway, B7.1 (CD80) or B7.2 (CD86) on the surface of antigen presenting cells delivers a constimulatory signal by interacting with CD28 found on the surface of resting T cells. These two costimulatory pathways are necessary regulators of T cell-dependent immune responses.

Reports indicate that treatment with molecules which block these two pathways, CTLA4/Ig and anti-CD154 antibodies, resulted in the prolonged graft suvival in several transplant models (272-276). Recently, Alan Kirk and David Harlan have shown that anti-CD154 antibodies bind to CD154, preventing the subsequent receptor binding and ultimately rejection. This treatment has prevented rejection of MHC mismatched kidney and pancreatic islet cell transplants in monkeys (277). It is interesting to speculate that PSGs may be functioning by a mechanism similar to anti-CD154 *in utero*. The ability of the semi-allogeneic fetus to escape immune rejection by the maternal immune system could be mediated by PSG binding to CD40 or B7 on the surface of macrophages, preventing the activation and development of allo-specific T cells. Further evidence that PSGs may perform such a function is provided by the fact that both PSGs and anti-CD154/CTLA4-Ig are associated with a predominating Th2 type response (272). In both transplantation and pregnancy, it seems that suppression and the Th1 pathway and stimulation of the Th2 pathway are necessary to achieve tolerance of the foreign tissue.

The concept of macrophage reprogramming may also explain the ability of the fetus to escape rejection by the maternal immune system. Macrophages are the one of the primary cellular sources of cytokine production in response to foreign antigens such as endotoxin (228). LPS-dependent activation of macrophages results in the secretion of proinflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-12, and proinflammatory cytokines, such as IL-10 and IL-4 (278). A recent

report suggests that certain microbial pathogens and malignant cells may trigger an imbalance in the expected proinflammatory response, attempting to evade host immunity (279). Their results indicate that LPS dependent modulation of TNF-α and NO phenotypes in macrophages depends on the signals mediated by specific soluble factor/mediators during LPS "priming" (279). In other words, exposure of macrophages to different cytokines alters a cell's responsiveness to LPS. The concept that cells may be preconditioned by their environment toward certain cytokine expression patterns can be applied to foreign fetal cells present within the uterus during pregnancy. The "primed" macrophages present within the uterus may be stimulated by the reciprocal production of IL-12 and IL-10 towards the development of a cytokine microenvironment which favors the Th1 or Th2 subset, respectively. PSGs may play a role in this type of macrophage reprogramming. Interaction with PSGs may result in the overproduction of IL-10 by macrophages and the subsequent development of a Th2 type immune response within the local environment of the uterus. Future research should be directed at investigating whether PSGs alter cytokine expression patterns of both LPS "primed" peritoneal macrophages as well as already activated uterine macrophages.

Another area that requires investigation is the basis of gender differences in autoimmune disease. The predominance of autoimmunity among women strongly suggests that hormones or other factors, specifically expressed by females, may have a role in modulating susceptibility to disease. High levels of progesterone have been shown to promote development of human Th2 cells (280).

Pregnant females exhibit a polarization of their immune system towards Th2 type cytokine profile (IL-10 and IL-4) which is associated the exacerbation of antibody mediated autoimmune diseases such as systemic lupus erythematosus (SLE), and the improvement of cell-mediated autoimmune disease such as multiple sclerosis (MS) and rheumatoid arthritis (RA) (100, 106). At the present time, it is unknown whether murine PSGs are found in the circulation or are capable of crossing the blood-brain barrier.

However, the ability of murine PSGs to upregulate the expression of IL-10 by murine macrophages suggests that they may have a role in generating an anti-inflammatory environment during pregnancy. The question of whether this anti-inflammatory state is local or systemic remains to be determined. Despite all of the unknowns, it is possible PSGs may have a role in regulating autoimmunity. Future research should utilize an *in vivo* system to investigate whether PSGs have the potential to improve cell-mediated or exacerbate antibody-mediated autoimmune diseases. Currently, there is a murine model available to study multiple sclerosis. Chronic relapsing experimental autoimmune encephalomyelites (EAE) in SJL mice is an autoimmune demyelinating disease of the central nervous system which exhibits many of the clinical as well as histopathologic symptoms of MS (281, 282). Using this system, it should be possible to examine whether PSGs are able to cross the blood-brain barrier to interact with the microglial cells and whether they function to improve the neurological condition of these mice.

Further characterization of the role of PSGs *in vivo* is necessary. Others in the laboratory are in the process of generating a transgenic mouse that overexpresses PSG18. This would be useful in both the mouse model of multiple sclerosis (MS) and the mouse model of immune pregnancy loss (CBA/J x DBA/2). In the mouse model of multiple sclerosis, PSG18 could be overexpressed, or recombinant PSG18 could be used to treat these animal. Success would be measured by improvement of the disease state. Also, it must be determined whether overexpression of PSG18 in female CBA/J mice results in a decreased fetal resorption rate. Again, this could also be done by injecting recombinant PSGs at certain days of gestation and examining the pregnancy outcome.

In conclusion, PSGs are a group of related pregnancy proteins which appear to directly regulate the innate arm of the immune system. The results from this study support the previously described theories that suggest that PSGs function to induce anti-inflammatory modulators of the immune system, thereby preventing rejection of the semi-allogeneic fetus.

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